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A Comparative Study of the Effects of Sesbania Drummondii on the Hepatic Cytochrome P-450 Monooxygenase Systems of Chickens and Rats.

Marcy Iva Banton

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**A comparative study of the effects of *Sesbania drummondii* on the
hepatic cytochrome P-450 monooxygenase systems of chickens
and rats**

Banton, Marcy Iva, Ph.D.

The Louisiana State University and Agricultural and Mechanical Col., 1990

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300 N. Zeeb Rd.
Ann Arbor, MI 48106

**A COMPARATIVE STUDY OF THE EFFECTS OF SESBANIA DRUMMONDII
ON THE HEPATIC CYTOCHROME P-450 MONOOXYGENASE SYSTEMS
OF CHICKENS AND RATS**

A Dissertation

**Submitted to the Graduate Faculty of the
Louisiana State University and
Agricultural and Mechanical College
in partial fulfillment of the requirements for the degree of
Doctor of Philosophy**

in

**The Interdepartmental Program in Veterinary Medical Sciences
(Toxicology Option)**

**by Marcy I. Banton
D.V.M., Louisiana State University, 1981
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ABSTRACT

The effect of an extract of Sesbania drummondii on the hepatic cytochrome P-450 monooxygenase system was evaluated in two experimental models, the White Leghorn chicken and the Sprague-Dawley rat. The cytochrome P-450 monooxygenase systems were compared using studies of catalytic activities and two major enzyme components, cytochrome P-450 and NADPH-cytochrome P-450 reductase. Two "classical" cytochrome P-450 inducers, phenobarbital and β -naphthoflavone, were used to probe induction mechanisms.

S. drummondii induced opposite effects on the hepatic cytochrome P-450 component of chickens versus rats. Cytochrome P-450 specific content was increased 2-fold in chickens but decreased 2-fold in rats. Qualitative differences in microsomal proteins were found using SDS-gel electrophoresis. The protein profile of S. drummondii-treated chickens was induced differently from phenobarbital or β -naphthoflavone. In the rat, the microsomal protein profile was similar to controls but of lower protein band staining intensity. Western blots revealed two S. drummondii-induced microsomal proteins of chickens that cross-reacted with antibody against rat P-450IA1. One of these proteins corresponded to the β -naphthoflavone-induced chicken protein. The other appeared unique to S. drummondii treatment.

Neither protein was detected by this antibody in the S. drummondii-treated rat.

Sesbania drummondii induced different responses in chicken versus rat cytochrome P-450 catalytic activities. Chickens showed increases of 2-fold in aminopyrine N-demethylase and 4-fold in aldrin epoxidase; no change in ethoxyphenoxazone and benzyloxyphenoxazone O-dealkylases; and a 2-fold decrease in pentoxyphenoxazone O-dealkylase. Similar activities were induced by phenobarbital. In contrast, S. drummondii-treated rats had 2 to 3-fold decreases in all activities, except ethoxyphenoxazone O-dealkylase, which was unchanged.

Species differences were also observed in the effect of S. drummondii on the NADPH-cytochrome P-450 reductase component. S. drummondii-treatment induced a 2-fold increase in NADPH-cytochrome c reductase activity in chickens but no change in treated rats. Use of cumene hydroperoxide indicated that the induced effects on the reductase contribute to aminopyrine but not aldrin metabolism in the chicken, and neither substrate's metabolism in the rat.

These findings suggest that Sesbania drummondii can induce changes in hepatic cytochrome P-450 monooxygenase systems and these changes are species-dependent.

CHAPTER 1

INTRODUCTION

Sesbania drummondii is a perennial herbaceous shrub or small tree inhabiting the sandy soils and marshes throughout the southeastern United States. This poisonous plant, also called rattlebrush or coffeebean, is sporadically ingested by livestock and usually results in fatalities (Kingsbury, 1964). Poisonings have been reported in cattle, sheep, and goats, usually in the fall or winter after other forage has become scarce (Marsh, 1920). The toxic principle of S. drummondii has not yet been identified, but appears to be concentrated in mature seed-containing pods (Terblanche et al., 1966).

Chickens and rats have been proven to be highly sensitive to extracts of S. drummondii seeds and pods and are, therefore, useful avian and mammalian experimental models for studying toxic effects of the plant (Flory and Hebert, 1984; Marceau-Day, 1988). Clinical signs, blood chemistry values, and tissue lesions have previously been described in poisoned chickens by Flory and Hebert (1984). Typically the clinical signs include weakness, depression, anorexia, diarrhea, ruffled feathers, cold feet, and rapid loss of body weight. An interesting clinical feature observed in chickens is a severe decrease in the concentration of total plasma proteins (Flory and Hebert, 1984). At necropsy, gross changes are

evident in body fat, muscle mass, liver, kidney, spleen, crop, intestines, and the heart (Flory and Hebert, 1984). Similar clinical signs and pathologic changes are observed in S. drummondii-poisoned rats (Marceau-Day, 1988).

Some of the changes in poisoned birds, as well as in rats, can be explained by inhibition of smooth muscle function. Venugopalan et al. (1984) reported significant decreases in the responsiveness of ileum and lung isolated from chickens poisoned with S. drummondii to two common smooth muscle agonists, histamine and carbachol. However, other changes in both species may be caused by toxic effects of S. drummondii on the liver. Earlier studies have focused on the liver to determine the cause of depleted circulating serum plasma concentration (Marceau-Day, 1988). These results indicate that S. drummondii inhibits protein synthesis and export protein release from the liver (Marceau-Day, 1988). Further investigations revealed that S. drummondii also alters a number of liver enzymes, including microsomal glutathione-S-transferase isozymes (Marceau-Day, 1988).

In addition to these toxic effects on the liver, there is the potential for S. drummondii to affect the major hepatic biotransformation system, the hepatic cytochrome P-450 monooxygenase system. Altering a major drug metabolizing system by consumption of S. drummondii could be important in

any species exposed to this toxic plant.

A related Sesbania species, Sesbania exaltata, is a major weed problem in soybean fields of the southeastern United States (Lunsford et al., 1976). There exists the potential for S. exaltata, S. drummondii, and other Sesbania species to contaminate harvested soybeans. The seeds from these plants could thereby be unintentionally and chronically consumed in soybean-containing products by both animals and humans.

Acute or chronic exposures to the Sesbania species, as with numerous xenobiotic compounds, could induce a host of undesirable consequences arising from effects on the microsomal cytochrome P-450-dependent mixed function monooxygenase system. Less toxic agents could become more toxic, producing acute intoxications on exposure. Alterations of this system could also change the pharmacokinetics and thus the efficacy of therapeutic agents given at the time of S. drummondii exposure. Additionally, S. drummondii induced changes in the cytochrome P-450 monooxygenase system could modify metabolism of endogenous substances resulting in reproductive and endocrine problems.

In order to lay groundwork to answer these questions, the effects of an extract of S. drummondii on the hepatic microsomal cytochrome P-450 monooxygenase system were studied.

CHAPTER 2

LITERATURE REVIEW

A. Sesbania drummondii

1. Botany of Sesbania:

Sesbania is a diverse group of plants that occur in both annual and perennial forms in tropical and subtropical regions and are adaptable to a wide variety of soil conditions. Sesbania drummondii is a perennial herbaceous shrub or small tree that inhabits the gulf coast plain of the southeastern United States south into Mexico (Lasseigne, 1973). The plant is locally very abundant from June to October and prefers soils that are the edges of low woods, banks of bayous, marshes, roadsides, margins of ponds, edges of ditches, railroad embankments, prairies, and sandy beaches (Lasseigne, 1973). S. drummondii is a common weed of south Louisiana pastureland and is also sometimes found around the periphery of soybean fields (Nicholson, 1989).

The genus Sesbania is taxonomically classified in the kingdom, Plantae; phylum, Spermatophyta; class, Angiospermae; subclass, Dicotyledonae; order, Rosales; family, Leguminosae; subfamily, Papilionoideae; and the tribe Robinieae (Kingsbury, 1964; Evans and Rotar, 1987). At least four subgenera, consisting of approximately 50 species, comprise the genus Sesbania and

include Sesbania, Agati, Glottidium, and Daubentonia (Evans and Rotar, 1987). Sesbania drummondii (Rydb.) Cory is placed in the subgenera Daubentonia.

Several latin and common names have been used by various authors to identify this species. S. drummondii is the same as Daubentonia longifolia, Daubentonia drummondii, and Sesbania cavanillesii (Kingsbury, 1964; Marsh and Clawson, 1920). Common names for this plant include rattlebrush, rattlebox, coffee bean, senna-bean, and poison bean (Ellis, 1975; Correll and Johnston, 1970).

Morphology among the Sesbania species is somewhat variable with similarities in the leaves, flowers, and seedpods. The S. drummondii shrub grows to a maximum height of approximately 6 meters (Ellis, 1975). The branches often die back during the winter with only the lowest part of the plant remaining alive and becoming woody (Correll and Johnston, 1970). The anatomical features of S. drummondii are presented in Figure 1. The leaves are pinnate, approximately 13 to 20 centimeters long, and are made up of 20 to 50 small green leaflets that are 15 to 35 millimeters long and 4 to 7 millimeters broad (Ellis, 1975; Correll and Johnston, 1970). The flowers are yellow, 13 to 16 millimeters long, and are formed in loose racemes (Ellis, 1975; Correll and Johnston, 1970). The seedpod is four-sided with four wings running the



DRUMMOND RATTLEBOX
Sesbania drummondii (Rydb.) Cory

Figure 1. Line drawing of Sesbania drummondii (Vines, 1960; reproduced with permission of University of Texas Press).

length of the pod and often reaches 5 to 6 centimeters in length (Ellis, 1975; Correll and Johnston, 1970). The seedpod changes color as it matures from green, to yellow, and finally to dark brown (Ellis, 1975). Four to seven small, hard seeds separated by transverse partitions are contained in the pod (Kingsbury, 1964; Ellis, 1975). The seeds are loose in the mature pods and rattle when the bush is in motion; hence the name "rattlebush" (Correll and Johnston, 1970).

2. Sesbania in agriculture:

The Sesbania species have both beneficial and harmful agricultural roles. Among the most important beneficial uses of the plants are in nitrogen fixation (green manures) and soil reclamation (Evans and Rotar, 1987). Because Sesbania species are well tolerant to soil salinity and alkalinity, flooding, and waterlogging, they are often used as green manures for rice and other crops in south and southeast Asia (Evans and Rotar, 1987). These tolerances also enable the Sesbania species to colonize problem soil sites and help in stabilizing and reclaiming these lands.

Several minor agricultural uses of the different species include pulp fiber resources, fuel wood, animal fodder, gum sources, and windbreaks (Evans and Rotar, 1987). Sesbania bispinosa, S. sesban, and S. grandiflora are the species of principal interest as commercial pulp fiber crops (Evans and

Rotar, 1987). Gums which have potential value in food processing and other industrial purposes are produced by many species of Sesbania. Sesbania grandiflora, S. formosa, and S. sesban are noted for their gum bark and S. bispinosa and S. sesban for their seed gums (Evans and Rotar, 1987).

Some of the Sesbania species have limited use as animal and human food (Evans and Rotar, 1987). The Sesbania species considered as potential sources of animal feed include S. bispinosa, S. sesban, and S. grandiflora.

The benefits of Sesbania in agriculture have been appreciated mostly outside of the United States. The predominant impact of the Sesbania species in American agriculture is negative.

The Sesbania species as weeds compete with crops for light, moisture, and nutrients. Sesbania seeds may contaminate harvest grain and reduce its value (Evans and Rotar, 1987). The Sesbania species with the worst weed reputation is S. exaltata. This plant is a widespread weed of crops in the southern United States, especially in soybean fields (Lunsford et al., 1976).

While the majority of the species of Sesbania are considered to be nontoxic, a few species are considered to be potentially toxic plants for grazing farm animals (Williams, 1983).

Toxicoses have been reported with S. vesicaria, S. punicea, and S. drummondii of the subgenera Daubentonia and Glottidium (Evans and Rotar, 1987).

3. Sesbania in medicine:

The Sesbania species have been medically useful to native healers and may become important in modern medical treatment as well. The herbal medicinal powers of the Sesbania species have been utilized by Asian cultures to treat a variety of disorders (Evans and Rotar, 1987). Extracts, mostly from S. grandiflora, S. sesban, and S. bispinosa, have been used as astringents, antipyretics, laxatives, anthelmintics, antifertility agents, and abortifacients (Evans and Rotar, 1987).

Some of the Sesbania species show potential for use in modern treatment regimens for cancer. Seed extracts of Sesbania vesicaria, S. punicea, and S. drummondii exhibit antitumor activity against lymphocytic leukemia P-388 (PS) in vivo (Powell et al., 1976). Efforts to isolate the antitumor agent in S. drummondii resulted in the identification of two inactive compounds, sesbanine and drummondol (Powell et al., 1979; Powell and Smith, 1981) and finally the active compound sesbanimide (Powell, 1983; Mayberry, 1983). Sesbanimide is a structurally unusual compound consisting of 3 rings linked by single bonds (Powell, 1983). The structure partly resembles

that of the antibiotics cycloheximide, streptimidone, and streptovitacin (Mayberry, 1983). This resemblance of sesbanimide to some antibiotics produced by soil bacteria raises a question as to its true source, the Sesbania plant or its associated microflora (Mayberry, 1983). Sesbanimide has demonstrated potent antitumor activity at very low doses in mice with leukemia and human carcinoma cells growing in vitro (Mayberry, 1983). A major obstacle to continued studies of sesbanimide is the very small concentration of the material in the seeds. Recent efforts are directed toward synthetic production of sesbanimide (Tomioka and Koga, 1984).

4. Sesbania toxicosis:

Toxicosis from S. vesicaria, S. punicea, and S. drummondii have been recognized in a variety of domestic animals since the early 1900's. Poisoning by these species generally occurs when animals are introduced onto new pastures and graze indiscriminately or during the winter months when other forage is scarce (Evans and Rotar, 1987). The Sesbania species pods remain on the plant during the winter months and present a hazard for hungry livestock.

Natural poisoning from S. vesicaria (Glottidium vesicarium) has been observed in sheep and cattle (Broughton and Hardy, 1939; Simpson and West, 1953; Newberne, 1953). Experimental studies in a number of animals have confirmed the ability of

this plant to produce illness and death. Emmel (1935) reported that 200 seeds of G. vesicarium fed to adult White Leghorn hens were fatal. Duncan et al. (1955) observed that seeds but not leaves of S. vesicaria were toxic to chickens. Similarly, Broughton and Hardy (1939) found the minimum lethal dose of seeds in sheep to be 0.0625% of body weight. Featherly et al. (1943) produced illness with G. vesicarium seeds in a cow, a rabbit, and in White Leghorn hens. Experimental observations of G. vesicarium-poisoned cattle were made by Emmel (1935) and Simpson and West (1953). Simpson and West (1953) observed dose related acute vs chronic effects from G. vesicarium seeds in steers.

Poisoning from S. punicea (Daubentonia punicea) has been reported in several domestic animals and poultry. Duncan et al. (1955) observed illness and death in chickens fed green or mature seeds of D. punicea. Shealy and Thomas (1928) found that as few as nine Daubentonia seeds were fatal to poultry. Terblance et al. (1966) observed experimentally induced S. punicea toxicosis in cattle, sheep, horses, pigs, dogs, rabbits, guinea pigs, chickens, pigeons and turkeys. Toxicosis, however, could not be produced in either young or adult ducks. The resistance in the duck was hypothesized to be related to the speed at which the seeds passed whole through the gastrointestinal tract.

Widespread losses in sheep and goat herds have been reported from indiscriminate grazing on S. drummondii (Marsh, 1920; Marsh and Clawson, 1920; Robey, 1925). Robey (1925) reported as many as 200 to 250 sheep and goats died after grazing the plant that was growing along the road down which they were driven. Marsh and Clawson (1920) experimentally examined the effects of D. longifolia seeds, pods and leaves in sheep. They found the seed and pods to be more toxic than the leaves and that as little as 30 grams of seeds were capable of poisoning and 60 grams killing sheep. Flory and Hebert (1984) evaluated the clinical and pathologic changes with extracts of S. drummondii on the chicken and found doses of 1.0 % of body weight to be uniformly lethal. Marceau-Day (1988) further characterized the clinical effects of S. drummondii extract in both the chicken and the rat. One human fatality of a small boy occurred following the ingestion of S. drummondii seeds (Ellis, 1975).

The clinical signs and necropsy changes in poisoned animals are indistinguishable between S. vesicaria, S. punicea and S. drummondii. Clinical changes in the gastrointestinal, nervous, cardiovascular and respiratory systems are consistently observed in Sesbania-poisoned animals. Typically, with acute poisoning the animal exhibits depression, weakness, anorexia, diarrhea, an irregular pulse, and decreased respiration. Coma usually precedes death (Ellis,

1975). Additionally, ruffled feathers (Flory and Hebert, 1984; Shealy and Thomas, 1928; Terblanche et al., 1966) and cold feet (Flory and Hebert, 1984) have been observed in poisoned chickens. With chronic low level exposures, poisoned animals are emaciated, sluggish, and have roughened hair or ruffled feathers and diarrhea. Natural recoveries are rare with acute or chronic exposures to Sesbania.

Postmortem examination of Sesbania-poisoned animals reveal changes of the cardiovascular, gastrointestinal, musculoskeletal, and urinary systems. Typical macroscopic lesions observed are muscle mass reduction, large prominent superficial blood vessels, organ congestion, flaccid heart in diastole and gastroenteritis of varying degrees of severity (Shealy and Thomas, 1928; Simpson and West, 1953; Emmel, 1935; Emmel, 1944). S. drummondii-poisoned chickens also have crops engorged with clear fluid, gizzards full of food, and empty gas-filled intestines (Flory and Hebert, 1984). Full crops and gizzards were also observed by Emmel (1935) in G. vesicarium-poisoned chickens. Less frequently observed lesions have included hemorrhagic lymph nodes in S. vesicaria-poisoned sheep (Broughton and Hardy, 1939), conjunctival exudation and eyelid edema in S. punicea-poisoned rats (Terblanche et al., 1966), and hyperemia around the edges of the eyelids in rabbits treated with S. punicea (Terblanche et al., 1966).

Microscopic descriptions of Sesbania-induced lesions are few in the literature and mostly confirm the gross findings. Differences, however, are noted in the microscopic lesions of the gastrointestinal tract between the toxic Sesbania species. Severe gastrointestinal changes are normally present with S. vesicaria and S. punicea. Emmel (1935) observed inflammation and necrosis from the crop to the proximal small intestine in G. vesicarium-treated chickens. Congestion and inflammation in the abomasum and duodenum of a G. vesicarium-poisoned cow was also reported by Emmel (1944). Terblanche et al. (1966) observed species differences in the presence and severity of the gastrointestinal lesions with S. punicea. Marked hyperemia, hemorrhages, and in some cases necrosis were present in sheep, rabbits, chickens, turkeys, pigeons, and pigs. Most S. punicea-treated rats, however, were devoid of any gastrointestinal lesions. The absence of gastrointestinal lesions was also observed by Flory and Hebert (1984) with S. drummondii treatment of chickens. Marceau-Day (1988) observed only moderate hyperemia of the lower gastrointestinal tract in chickens and rats dosed with S. drummondii.

Several researchers noted microscopic changes in the kidney. Flory and Hebert (1984) observed glomerular contraction from the distended parietal layer of the glomerular capsule (Bowman's capsule) and the presence of protein in the convoluted tubules of S. drummondii-poisoned chickens.

Simpson and West (1953) reported that the pelvis of the kidney of a cow poisoned with G. vesicarium contained an albumin-like substance. Emmel (1944) reported that two cows administered G. vesicarium seeds had kidneys with generalized, well-advanced albuminous degeneration which was more marked in the convoluted rather than the straight tubules.

The clinical pathological features of Sesbania species poisoning have been documented only with S. punicea and S. drummondii. Terblanche et al. (1966) observed increases in blood glucose, blood urea nitrogen and creatinine in S. punicea treated sheep, cattle, and horses. Flory and Hebert (1984) reported increases in creatine kinase and plasma cholinesterase, marked decreases in total plasma protein concentrations, and no changes in packed cell volumes and plasma glucose concentrations in chickens administered S. drummondii. Marceau-Day (1988) more completely characterized the clinical pathological changes in S. drummondii-poisoned chickens and rats. She observed decreases in serum proteins, plasma cholinesterase, lactate dehydrogenase, gamma glutamyl transpeptidase, and alkaline phosphatase in both chickens and rats. Affected chickens, but not rats, also had decreases in packed cell volumes and serum potassium concentrations. Liver microsomal glutathione peroxidase activity was reduced in Sesbania-treated chickens whereas cytosolic glutathione peroxidase was increased in both treated chickens and rats.

Several glutathione-S-transferase isozymes were increased in the microsomal and cytosolic fractions of S. drummondii-treated chickens.

The pathophysiology and causative toxins of Sesbania-induced toxicosis are uncertain. Venugopalan et al. (1984) observed decreased responsiveness of intestinal and lung parenchymal strips to histamine and carbachol in S. drummondii-treated chickens indicating the likelihood of smooth muscle involvement as a cause of toxicosis. Additional studies by Venugopalan et al. (1987) revealed S. drummondii inhibition of vascular smooth muscle which may be the cause of the frequently observed postmortem finding of engorged blood vessels.

Several S. drummondii-induced toxic effects may contribute to the marked drop in total plasma protein concentration. Flory and Hebert (1984) suggested that toxic glomerular damage with leakage was partly responsible for the protein decrease. Marceau-Day (1988) demonstrated that the plasma protein depression in S. drummondii-poisoned chickens was influenced by decreases in protein synthesis and export protein release from the liver.

Attempts at isolating the toxic principle of Sesbania species have led to the discovery of a number of new compounds. Robey

(1925) described a sapotoxin from extracts of D. longifolia. Powell et al. (1976) isolated three compounds: sesbanine, drummondol, and sesbanimide. Whether any, all, or none of these compounds contribute to Sesbania-induced toxicosis is not presently known. However, the many documentations of pharmacological and toxicological responsiveness to ingestions of this plant suggest that classical phase I and/or phase II metabolic processes are involved in the manifestation of clinical signs.

B. Hepatic cytochrome P-450 monooxygenase system

Many foreign compounds, or xenobiotics, to which organisms are exposed in their environment are lipid soluble and readily gain entrance through biological membranes. These lipophilic compounds would tend to accumulate in the living organism and disrupt cellular processes if there was not a mechanism to convert them to more water soluble and readily excretable metabolites. The biotransformation enzymes are primarily responsible for this function (Sipes and Gandolfi, 1986).

The biotransformation enzymes have been divided into two types (Williams, 1959): Phase I and Phase II. The Phase I reactions are the predominate biotransformation pathway and involve oxidation, reduction, and hydrolysis. Phase I reactions result in functionalization which is the addition or uncovering of specific functional groups (deBethizy and Hayes, 1989). Phase II reactions are biosynthetic reactions where the foreign compound or Phase I-derived metabolite is covalently linked to an endogenous molecule, producing a conjugate (Sipes and Gandolfi, 1986).

The cytochrome P-450 monooxygenase system is the most important enzyme system involved in Phase I biotransformation. This system is a unique and versatile catalyst capable of metabolizing both lipophilic xenobiotics as well as a variety

of endobiotics such as steroids, prostaglandins, leukotrienes, and fatty acids (Gelboin, 1988).

The first description of cytochrome P-450 came in independent studies of microsomes from rat liver by Klingenberg (1958) and pig liver by Garfinkel (1958). Several years later, Omura and Sato (1962, 1964a, 1964b) showed that the pigment was a b-type of hemocytchrome and named it "P-450" because it exhibited a spectral Soret peak at 450nm when the cytochrome was reduced and bound to carbon monoxide. Cytochrome P-450 has been shown to be present in mammals, birds, reptiles, amphibians, insects, bacteria, yeasts, and higher plants (Nebert et al., 1989a).

1. Structure of the cytochrome P-450 monooxygenase system:

In mammals, the cytochrome P-450 system is most abundant in the liver but is also present in nearly all tissues except striated muscle and erythrocytes (Guengerich, 1988). Within the cell, these enzymes are membrane bound and are concentrated in the endoplasmic reticulum, the nuclear envelope, and the mitochondria (Guengerich, 1988). In general, the cytochrome P-450 system involved in xenobiotic biotransformation is located in the endoplasmic reticulum (microsomal), whereas those involved in steroid metabolism are in the mitochondria.

The major components of the microsomal cytochrome P-450 monooxygenase system are two enzymes: cytochrome P-450 and NADPH cytochrome P-450 reductase. Other components which may also be involved with metabolism of specific xenobiotics are cytochrome b5 and cytochrome b5 reductase (deBethizy and Hayes, 1989).

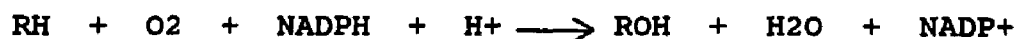
Cytochrome P-450 is not a single enzyme but a family of multiple forms or isozymes (Gibson, 1989). More than 60 different forms of cytochrome P-450 have been isolated from various sources (Guengerich, 1988). The rat liver alone contains 20 different forms which have been purified and characterized (Ortiz de Montellano, 1986). The availability of cDNA and some protein sequences has permitted the categorization of these cytochrome P-450 enzymes into gene families (Guengerich, 1988). The cytochrome P-450 enzymes are coded for by a "supergene" family, which contains 78 genes divided into 14 families (Nebert et al., 1989b).

2. Function of the cytochrome P-450 monooxygenase system:

The cytochrome P-450 monooxygenase system functions mostly in the oxidation and to a lesser degree the reduction of chemically diverse endogenous and exogenous substrates. The microsomal cytochrome P-450 oxidation of xenobiotics is concerned primarily with detoxication through the formation of more polar, biologically-inactive, readily excretable

metabolites (Ioannides and Parke, 1987). However, this system is also capable of the bioactivation of compounds to reactive intermediates which may interact with vital cellular macromolecules resulting in DNA damage, mutations, malignancy and other pathologic processes (Ioannides and Parke, 1987).

The overall oxidation reaction catalyzed by the microsomal cytochrome P-450 monooxygenase system and stoichiometry are illustrated by the following equation (deBethizy and Hayes, 1989):



where RH = substrate

NADPH = required cosubstrate

The generally accepted sequence of reactions involved in the oxidation of substrates is shown as a catalytic cycle in Figure 2. The initial step of the cycle is the binding of substrate to the oxidized form of cytochrome P-450 (Fe³⁺) to form a substrate-cytochrome P-450 complex. The complex is reduced by a single electron to form a ferrous (Fe²⁺) P-450 substrate complex. This electron is provided by NADPH via the flavoprotein, NADPH-cytochrome P-450 reductase. The reduced substrate P-450 complex binds to oxygen to form a ternary complex. The next stage of the catalytic cycle is not completely understood. The ternary complex accepts a second electron from either NADH or NADPH resulting in the activation

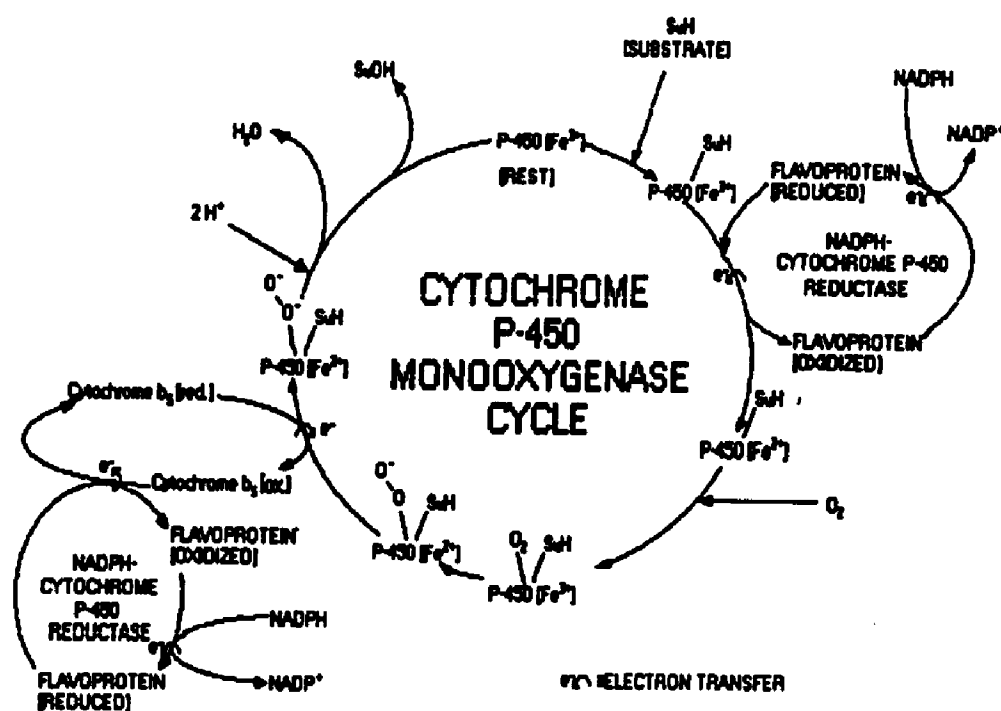


Figure 2. Catalytic cycle of the cytochrome P-450-dependent monooxygenase.

of the oxygen molecule. The activated oxygen is then inserted into the substrate forming an oxidized substrate with the release of water and regeneration of oxidized ferric cytochrome P-450 (deBethizy and Hayes, 1989).

A number of different types of oxidation reactions are catalyzed by the cytochrome P-450 monooxygenase system. These include aliphatic and aromatic hydroxylation, epoxidation, N-,O-and S-dealkylation, deamination, desulfuration, sulfoxidation, N-hydroxylation, and oxidative dehalogenation (Sipes and Gandolfi, 1986).

In addition to their physical differences, the cytochrome P-450 isozymes exhibit variable substrate and product specificity (Gelboin, 1988). The individual proteins exhibit overlapping substrate and oxygenation mechanism specificity, as well as varying degrees of selectivity for certain substrates and reactions catalyzed (Ioannides and Parke, 1987). Differences in substrate specificity, regiospecificity, and stereospecificity of various cytochrome P-450 isozymes play an important role in regulating the balance between bioactivation and detoxication pathways of a given xenobiotic (Lu, 1979). Thus the relative proportions of various forms of cytochrome P-450s in a given tissue and their regulation are important factors in determining the outcome of chemical biotransformation.

3. Regulation of the cytochrome P-450 monooxygenase system:

The distribution pattern of the various cytochrome P-450 isozymes is genetically determined by the predominance, or relative absence of certain isozymes (Ioannides et al., 1984). These differences contribute to the observed differences in how species metabolize xenobiotics. The isozymal distribution pattern can also be modified by factors such as age, nutrition and diet, and selective induction of cytochrome P-450 isozymes following previous exposure to chemicals (Ioannides et al., 1984).

The magnitude, onset and duration of induction of cytochrome P-450 isozymes are influenced by factors related to the inducing agent as well as to the host. The chemical nature of the inducing agent, its dose, and the duration of exposure all contribute to the enzyme induction. A number of different chemical agents have been found to induce the cytochrome P-450 monooxygenases.

The early literature described three types of inducers: phenobarbital (PB), 3-methylcholanthrene (3-MC), and mixed (Goldstein, 1980). These are considered the "classical" types of cytochrome P-450 monooxygenase inducers. Now, there are four other distinct major inducer categories and the list is likely to grow. These others are considered the "non-classical" inducers and include ethanol, steroids, hormones

and peroxisome proliferators (Guengerich, 1990). There are also P-450 inducers that do not fit into any of the above categories (Okey, 1990).

Much of the cytochrome P-450 induction study has been limited to the classical inducers. Phenobarbital induces the cytochrome P-450 isozyme family and 3-MC induces the cytochrome P-448 isozyme family. Consequently, the cytochrome P-450 isozyme family is referred to as the PB inducible form and the cytochrome P-448 isozyme family is referred to as the 3-MC inducible forms (Lewis et al., 1986). Other chemical inducers are classified as PB-like or 3-MC-like if their pattern of isozymal induction is similar to these two chemicals. Some chemicals are considered to be mixed-type inducers if characteristics of both PB and 3-MC are observed.

Several classes of cytochrome P-450 isozymes and their gene families have been related and classified according to their chemical inducing agent. In the rat, the major PB-inducible isozyme is referred to as cytochrome P-450b (P-450IIB1) and the major 3-MC-inducible isozyme is cytochrome P-450c (P-450IA1) (Nebert et al., 1989b). However, not all P-450 isozymes are inducible by xenobiotic chemicals. Rat liver P-450f and P-450g are relatively refractory to induction by all common classes of P-450 inducers (Bandiera et al., 1986). The steroid-metabolizing P-450's are resistant to xenobiotic

chemical induction; but instead their regulation often involves induction by endogenous substances (Okey, 1990).

Marked physiologic and anatomic changes are observed in liver cells following pretreatment with PB and 3-MC. Varying degrees of liver enlargement and smooth endoplasmic reticulum proliferation are observed as well as changes in concentration of the cytochrome P-450 enzyme components and the levels of certain cytochrome P-450-mediated enzyme activities (Sipes and Gandolfi, 1986).

Some of the cytochrome P-450 mediated enzyme activities show varying degrees of specificity for the cytochrome P-450 isozyme families and therefore have been used to indirectly measure the major types and degrees of induction. In the rat, the enzyme activity arylhydrocarbon hydroxylase has been associated with induction of cytochrome P-448 (McCormack et al., 1979) and aminopyrine N-demethylase has been associated with induced cytochrome P-450 (Kato and Onado, 1970). Unfortunately, many of these activities have not been found to clearly distinguish between the cytochrome P-450 isozyme families. Recently, however, cytochrome P-450 mediated enzyme activities that show a high degree of isozyme specificity have been reported. In the rat, the O-dealkylation of various substituted alkoxyresorufin analogues have provided a simple routine test for distinguishing between several inducible

isozymes of cytochrome P-450 (Burke and Mayer, 1983; Burke et al., 1985).

The most common mechanism for cytochrome P-450 monooxygenase induction involves transcriptional activation of the gene (Nebert et al., 1989a). This appears to be the case with PB, 3-MC, and most other inducing chemicals. However, ethanol, pyrazole, imidazole, and acetone induction, have been shown to involve post-transcriptional and post-translational gene regulation (Nebert et al., 1989a).

Not only can cytochrome P-450 monooxygenase activity be increased through chemical interaction, it can be decreased as well. Inhibition of cytochrome P-450 mediated xenobiotic biotransformation can result from a number of general and specific inhibitory mechanisms. The general or non-specific inhibitors affect cytochrome P-450 as well as the majority of biotransformation reactions. These include agents that affect protein synthesis, compete for active sites or cofactors of the enzymes, cause allosteric changes in enzyme conformation, interfere with transport components in multienzymic systems and cause loss of functional tissue (Sipes and Gandolfi, 1986). A number of chemicals have more specific inhibitory effects on the cytochrome P-450 system. Ethylisocyanide and carbon monoxide compete with molecular oxygen by interacting with the reduced heme moiety of cytochrome P-450. Metirapone,

SKF-525A, piperonyl butoxide, and others compete for the substrate binding site of cytochrome P-450. Some of these inhibitors are selective for certain of the cytochrome P-450 isozyme families. These chemicals can produce either a competitive or a noncompetitive inhibition of cytochrome P-450. Another group of chemicals act as suicide inhibitors. These chemicals are activated by cytochrome P-450 to reactive metabolites which then covalently bond to the heme moiety resulting in destruction of the heme and loss of cytochrome P-450 activity (Sipes and Gandolfi, 1986).

Clearly, the presence of chemical inducers and inhibitors can profoundly alter the biotransformation capabilities of the cytochrome P-450 monooxygenase system.

4. Avian hepatic cytochrome P-450 monooxygenase system:

The avian hepatic cytochrome P-450 monooxygenase system has many similarities to the mammalian system with regard to structure, function, and regulation. However, very specific differences between the two classes have been noted in cytochrome P-450 content, cytochrome P-450 mediated enzyme activities, and response to inducers (Ronis and Walker, 1989). In addition, qualitative and quantitative differences are also evident among the avian species (Pan and Fouts, 1978).

The physiological and toxicological significance of the cytochrome P-450 monooxygenase system of birds has been recognized only recently. Studies have linked the decline of predators such as the peregrine (Falco peregrinus) and sparrowhawk (Accipiter nisus) in Great Britain and the bald eagle (Haliaeetus leucocephalus) in North America to the bioaccumulation of organochlorine insecticides, the likely consequence of slow metabolism (Walker and Stanley, 1987; Walker, 1981). Certain of the avian cytochrome P-450 isozymes have been shown to be readily inducible by environmental chemicals, thus these may be useful as biological monitors for exposures in the field (Ronis and Walker, 1989). Knowledge of the role of the monooxygenases in the food producing birds, chickens, ducks, turkeys, and geese, is important for understanding the safe and effective use of xenobiotics in these species (Ronis and Walker, 1989; Dalvi et al., 1987).

The concentration of cytochrome P-450 in liver microsomes of birds is, on average, four-fold less than mammals (Ronis and Walker, 1989; Dalvi et al., 1987). The Passeriformes and Galliformes have the highest of the avian cytochrome P-450 contents, whereas the Pelecaniformes and Charadriiformes, fish eating birds, have the lowest content (Ronis and Walker, 1989). Among the food producing avian species, turkeys have the highest cytochrome P-450 content followed by geese, chickens, ducks, and quail (Dalvi et al., 1987). Riviere et

al. (1985) observed that the cytochrome P-450 content in duodenal microsomes in the grey partridge and chicken were very similar in concentration to those in liver. The Japanese quail and pheasant, however, had extremely low duodenal cytochrome P-450 contents (Riviere et al., 1985).

The activity of the hepatic cytochrome P-450 monooxygenases in birds has been measured using a variety of lipophilic substrates (Ronis and Walker, 1989). The relative activity in the avian species can be compared to the activity in male rats by adjusting for liver to body weight ratios (Walker, 1978 and 1980). Differences in these activities appear to be a function of the substrate used. The cytochrome P-450 mediated metabolism of aldrin, aminopyrine, and HCE (an analogue of dieldrin) are relatively low in birds compared to the male rat (Walker and Ronis, 1989). Alternatively, the relative activity for aniline metabolism is similar and that for 7-ethoxyresorufin greater for birds than the rat (Walker and Ronis, 1989). A comparison of the relative activities for many substrates, with the exclusion of ethoxyresorufin, against the body weights in mammals and birds show that most birds have activities substantially below those of mammals of similar body weights (Ronis and Walker, 1989). These data also show a distinct difference between predator bird species and non-predator birds. Predator birds show lower relative activities than non-predators (Ronis and Walker, 1989). As is

true for the cytochrome P-450 content, the Passeriformes and Galliformes show high relative activities compared to other bird orders (Ronis and Walker, 1989). The more chemically complex diet of these herbivorous and omnivorous birds may contribute to this finding (Ronis and Walker, 1989).

The hepatic cytochrome P-450 monooxygenases of birds, like those of mammals, are inducible but with a number of significant differences. The 3-MC-type of cytochrome P-450 inducer has been shown to produce increases in all the avian species tested. These include chickens, Japanese quail, and pigeons treated with either 3-MC, β -naphthoflavone (BNF), or 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) (Ronis and Walker, 1989). Buynitzky et al. (1978) administered 3-MC to White Leghorn chickens and observed a 4-fold increase in cytochrome P-450 content, 1.7-fold in ethylmorphine N-demethylase, 2.5-fold in aniline hydroxylase, and 20-fold in arylhydrocarbon hydroxylase. Ehrich et al. (1984) observed increased detoxication of the organophosphate malathion in livers of chickens given BNF and 3-MC. Ehrich and Larsen (1983) detected increases in cytochrome P-450 content, p-nitroanisole O-demethylase, and aniline hydroxylase in BNF and 3-MC treated adult White Leghorn hens.

Consistent findings observed with 3-MC induction in both birds and mammals are increases in cytochrome P-450 content,

arylhydrocarbon hydroxylase activity, and the characteristic blue shift in the reduced cytochrome P-450-CO difference spectrum from 450 nm to 448 nm. Some features that are different between the two classes, however, include: more marked increases in overall cytochrome P-450 content in birds than in mammals; earlier development of inducibility in the avian embryo than the mammalian embryo; and increases in the enzyme activities aminopyrine N-demethylase, ethylmorphine N-demethylase, and biphenyl-2-hydroxylase in birds but not in mammals (Ronis and Walker, 1989). In addition, the Ah receptor observed in 3-MC induced mammals has not been identified in birds which may indicate that 3-MC induction is mediated differently between the two species (Sawyer et al., 1986).

Phenobarbital-type induction has produced variable responses in birds (Walker and Ronis, 1989). Chickens, as do mammals, show good response whereas the Japanese quail and the cormorant show little or no response to PB induction (Walker and Ronis, 1989). Strittmatter and Umberger (1969) administered PB to several age groups of chickens and in all stages of development observed increases of cytochrome P-450 content and meberal N-demethylase activity. Powis et al. (1976) observed three- to four-fold increases in cytochrome P-450 content with increases in aminopyrine N-demethylase and aniline hydroxylase in 7-day old chickens treated with PB.

Pilch and Combs (1981) studied the relationship of dietary vitamin E and selenium on PB induction in young male and female chickens. They found maximal induction was impaired in male but not female chicks by combined vitamin E/selenium deficiency. Lorr and Bloom (1987) examined the ontogeny of the chicken cytochrome P-450 enzyme system from 7 days of incubation to 36 days post hatching. They observed increases in cytochrome P-450 content and aminopyrine N-demethylase activity as early as 7 days of incubation. The early response seen by several investigators in birds to PB induction has not been observed in mammals (Ronis and Walker, 1989).

Immunochemical identification of avian cytochromes P-450 using monoclonal antibodies raised against PB isoforms of rat cytochrome P-450 give additional evidence of difference among bird species. A cytochrome P-450 was purified from PB-treated chick embryos and shown to cross react with antibodies raised to rat cytochrome P-450b (P-450IIB1) (Brooker et al., 1983). However, cormorants dosed with PB did not have a cytochrome P-450 that cross reacted with rat cytochrome P-450b (P-450IIB1) (Ronis and Walker, 1989).

Another difference between birds and mammals is the number of phenobarbital inducible genes present in the genome. Mattschoss et al. (1986) reported the presence of two independent PB inducible P-450 genes in the chicken genome.

Hobbs et al. (1986) used the chicken cytochrome P-450 protein sequence to propose that the ancestral genes of mammals should have at least four distantly related PB-inducible gene subfamilies.

Mixed-type induction occurs in birds as in mammals with exposure to a number of complex organic compounds including polychlorinated biphenyls, polybrominated biphenyls, hexachlorobenzene, and 2-acetylaminofluorene (AAF) (Ronis and Walker, 1989). These studies have mostly been limited to the chicken and quail. Cecil et al. (1978) reported that Aroclor 1254 and 1268 but not 1242 were potent inducers of hepatic enzyme activity in White Leghorn cockerels. Ehrich and Larsen (1983) observed that Aroclor 1254 was a selective inducer of aniline hydroxylase activity in hens. Differences in induction between chicks and young barn owls treated with Aroclor 1254 were observed by Rinzky and Perry (1983). Darby et al. (1985) examined AAF treatment in chick embryos and found induction similarities to both PB and 3-MC.

A number of other classes of compounds produce induction responses in birds that are not characteristic of either PB-, 3-MC- or mixed-type of inducers. One interesting class of compounds is the ergosterol biosynthesis inhibitors, prochloraz, imazalil and ketaconazole. Prochloraz is a potent inducer of cytochrome P-450 in birds but only a weak inducer

in rats (Riviere, 1983; Rivere et al., 1985). In the red-legged partridge, prochloraz induces a ten-fold increase in cytochrome P-450 content, a substantial increase in ethoxyresorufin O-deethylase and aldrin epoxidase activities, and a shift in the cytochrome P-450-CO-difference spectrum to a peak at 453 nm (Johnston et al., 1989). Immunochemical studies of the partridge show that the cytochrome P-450 form induced by prochloraz cross reacts with antibodies against cytochrome P-450c of the rat (Johnston et al., 1989). These changes give strong indications of 3-MC-type of induction as well as another new type of induction (Johnston et al., 1989). Riviere et al. (1983) studied the effect of pyrethroids on mixed function oxidase activity in Japanese quail. Small increases in P-450 specific content and ethoxyresorufin O-deethylase activity were observed in the duodenum, however there were no significant changes in the liver.

The glucocorticoids also appear to be inducers of hepatic mixed function oxidase activity in the chick embryo. Leahey et al. (1979) observed increases in cytochrome P-450 specific content and aminopyrine N-demethylase activity in chick embryos administered corticosterone.

The response of the avian cytochrome P-450 monooxygenase system to inhibitors has not been thoroughly investigated. However, it is likely that the avian cytochrome P-450 system

responds similarly to the general and specific inhibitors of the mammalian cytochrome P-450 system. Two insecticides have been found to inhibit avian cytochrome P-450. Malathion treatment resulted in significant inhibition of aniline hydroxylase and the demethylation of p-chloro N-methyl aniline in White Leghorn cockerels (Varshneya et al., 1986). DDT caused decreased aniline hydroxylase activity in Japanese quail and chickens (Sell et al., 1971 and 1972; Sifri et al., 1975) and inhibition of aminopyrine N-demethylase activity in quail (Sifri et al., 1975). However, ducklings given DDT did not show suppressed microsomal monooxygenase activities (Sifri et al., 1975). This demonstrates that there are differences between avian species in the response to inhibitors as well as to inducers of the cytochrome P-450 monooxygenase system.

CHAPTER 3

EXPERIMENTAL GOALS AND DESIGN

The primary objective of this study was to determine the effects of treatment with an extract of Sesbania drummondii on the major biotransformation system, the hepatic cytochrome P-450 monooxygenase system. Comparisons were sought between the avian experimental model for Sesbania drummondii toxicosis, the chicken, and the mammalian experimental model, the rat. This study included 4 experimental phases.

A. Phase I - Preliminary investigations of the effects of an extract of *Sesbania drummondii* on hepatic cytochrome P-450 monooxygenase systems of chickens and rats.

The preliminary investigations were designed to determine fundamental effects of an extract of Sesbania drummondii on the hepatic cytochrome P-450 monooxygenase systems of chickens and rats. This was accomplished with 4 experiments.

The purpose of experiment 1 was to characterize and compare the effects of Sesbania drummondii-treatment on components of the hepatic cytochrome P-450 monooxygenase system in treated chickens and rats vs. untreated controls. Evaluations were made of liver to body weight ratio, cytochrome P-450 and b5 content, NADH- and NADPH-cytochrome c reductase activity and

six selected cytochrome P-450 mediated enzyme activities.

Experiment 2 was designed to evaluate the effects of the Sesbania drummondii-extraction buffer on the cytochrome P-450 monooxygenase system. Assurances were needed that changes observed were due to Sesbania drummondii alone and not the extraction solution. These were obtained by determining the extraction solution effects on the cytochrome P-450 content and two cytochrome P-450-mediated enzyme activities in chickens and rats treated with the extraction buffer vs. untreated chicken and rat controls.

Experiment 3 determined whether the Sesbania drummondii-induced effects on the hepatic cytochrome P-450 system in rats were sex-dependent. This information was necessary for the design of subsequent studies. A comparison between the cytochrome P-450 content of Sesbania drummondii-treated male and female rats was used to satisfy this goal.

Experiment 4 evaluated the contribution of changes in microsomal membrane stability to the Sesbania drummondii-induced effects of the hepatic cytochrome P-450 monooxygenase system. This was accomplished by measurements of a microsomal membrane marker enzyme, glucose-6-phosphatase.

B. Phase II - Characterization of *Sesbania drummondii* effects on catalytic properties of the hepatic cytochrome P-450 monooxygenase systems. Comparison with effects caused by phenobarbital and β -naphthoflavone.

The goal of the catalytic study was to characterize the effects of an extract of *Sesbania drummondii* on cytochrome P-450-mediated enzyme activities of treated chickens and rats and to compare these changes with those induced by the 2 major classes of cytochrome P-450 inducing agents, phenobarbital and β -naphthoflavone. This was achieved by characterizing cytochrome P-450 mediated enzyme activities of both low and high degrees of specificity for cytochrome P-450 isozyme families. The enzyme activities studied were aminopyrine N-demethylase, aldrin epoxidase, and the alkoxyphenoxazone O-dealkylases. Aminopyrine N-demethylase activity has been associated with the phenobarbital-inducible cytochrome P-450 isozyme subfamily (P-450IIB1), although the specificity of this activity is low (Lubet *et al.*, 1985). The aldrin epoxidase activity has also been associated with phenobarbital-inducible cytochrome P-450 but with greater specificity (Lubet *et al.*, 1985). Presently, the O-dealkylation of various alkoxyphenoxazone derivatives are the most specific of the cytochrome P-450-mediated enzyme activities for distinguishing between the inducible cytochrome

P-450 isozymes in rat hepatic microsomes. Increases in the O-dealkylation of pentoxy- and benzyloxyphenoxazone are associated with induced rat cytochrome P-450IIB1 and an increase in the O-dealkylation of ethoxyphenoxazone is associated with induction of cytochrome P-450IA1 (Burke and Mayer, 1983; Burke et al., 1985).

C. Phase III - Effects of *Sesbania drummondii* on NADPH-dependent cytochrome P-450 reductase. Comparison with effects caused by phenobarbital and β -naphthoflavone.

The purpose of the reductase study was to determine whether the *Sesbania drummondii*-induced changes in the cytochrome P-450 monooxygenase system were enzymic in nature or were the result of altered efficiency of electron transfer. This was accomplished by characterizing the effects of *Sesbania drummondii* on the NADPH-dependent cytochrome P-450 reductase of the cytochrome P-450 monooxygenase system and to compare these changes to those induced by phenobarbital and β -naphthoflavone. NADPH-dependent cytochrome P-450 reductase was characterized by measuring the reductase activities and by determining the relative contribution of the reductase component to the overall changes induced in the cytochrome P-450 system. The reductase activity was determined by measuring the rates of reduction of cytochrome c. The

relative contribution of the reductase component to the overall changes induced in the cytochrome P-450 system was determined by comparing reductase-dependent, NADPH-mediated metabolism to reductase-independent, organic hydroperoxide-mediated metabolism i.e., the peroxygenase activity of cytochrome P-450. Two cytochrome P-450-mediated activities, aminopyrine N-demethylase and aldrin epoxidase were measured via the peroxygenase activity using two organic hydroperoxides, cumene hydroperoxide and t-butyl hydroperoxide as oxygen donors. These activities were characterized in both treated chickens and rats.

D. Phase IV - Induction of microsomal enzymes by *Sesbania drummondii* as determined by SDS-PAGE and western blots with anti-cytochrome P-450 antibodies. Comparison with effects caused by phenobarbital and β -naphthoflavone.

Electrophoretic and immunochemical characterization of cytochrome P-450-induced changes provides for definitive classifications as to cytochrome P-450 isozyme family. Therefore, the final study was designed to compare the *Sesbania drummondii*-induced changes to those of phenobarbital and β -naphthoflavone using SDS gel electrophoresis and Western blotting techniques.

CHAPTER 4
SPECIFIC OBJECTIVES, MATERIALS AND METHODS,
RESULTS AND DISCUSSION

A. PHASE I - PRELIMINARY INVESTIGATIONS OF THE EFFECTS OF AN EXTRACT OF SESBANIA DRUMMONDII ON THE HEPATIC CYTOCHROME P-450 MONOOXYGENASE SYSTEMS OF CHICKENS AND RATS.

EXPERIMENT 1 - Initial characterization studies of the effects of an extract of Sesbania drummondii on hepatic cytochrome P-450 monooxygenase systems of chickens and rats.

OBJECTIVES:

1. To determine the effect of treatment with S. drummondii on body and liver weight in chickens and rats.
2. To determine the effect of S. drummondii-treatment on hepatic microsomal cytochrome P-450 and cytochrome b5 specific content in chickens and rats.
3. To determine the effect of S. drummondii-treatment on hepatic microsomal NADH-and NADPH-dependent cytochrome c reductase activity in chickens and rats.
4. To determine the effect of S. drummondii-treatment on selected hepatic microsomal cytochrome P-450 mediated-activities.

MATERIALS AND METHODS:

Many of the materials and methods described below will be used in subsequent studies throughout this project.

A. Chemicals:

4-Aminophenol (99% pure), aniline (99% pure), benzo(a)pyrene (98% pure), 4-dimethylaminoantipyrene (98% pure), 7-ethoxycoumarin (99% pure), and umbelliferone (98% pure) were purchased from Sigma Chemical Co. (St. Louis, MO). 7-Ethoxyresorufin (98% pure) was purchased from Molecular Probes, Inc. (Eugene, OR). Resorufin (75% dye content) was obtained from Aldrich Chemical Co., Inc. (Milwaukee, WI). Aldrin (99.4% pure) and dieldrin (99.8% pure) were obtained from the EPA (Research Triangle Park, NC). 3-Hydroxybenzo(a)pyrene (99% pure) was purchased from the Chemical Repository of the National Cancer Institute (Bethesda, MD). All other chemicals and biochemicals were of analytical grade or better and were obtained from various commercial sources.

B. Animals:

Twenty-four male and female 300-to 500-g White Leghorn chickens were obtained from the Department of Veterinary Science, Louisiana State University, Baton Rouge, LA. Eighteen male Sprague-Dawley 200-to 300-g rats were obtained from Hilltop Lab Animals, Inc. (Scottsdale, PA). The chickens

were group housed in commercial brooders and the rats were maintained 3 per cage on a 12-hr light/dark cycle in a temperature (21-24 C) and humidity (40-60 %) controlled environment. Chickens and rats were given feed (Purina chick Startena and Purina rat chow) and water ad libitum. Both species were acclimated for 1 week. Experiments using chickens were performed with 3 groups of 8 animals, 4 treated and 4 control in each group. Experiments using rats were performed with 3 groups of 6 animals, 3 treated and 3 control in each group.

C. Treatments:

Extracts of S. drummondii were prepared from dry ground seeds and pods gathered locally. Ground seeds and pods have virtually the same toxicity as whole ground seeds (Marceau-Day, 1988). The ground seeds and pods were soaked overnight in a 25 mM sodium bicarbonate buffer (pH 7.4) containing 0.02% sodium azide in a ratio of 1 part ground seeds to 5 parts buffer. Sodium azide was added to inhibit bacterial growth in the extract. The extract was recovered by vacuum filtration through 6 layers of cheese cloth. The concentration of the extract was calculated from the volume of filtrate per gram of seeds/pods. Animals were weighed daily for 3 days and then given either freshly prepared extracts of S. drummondii by gavage, or they received no treatment. Control animals were not given the extracts. No differences in the growth and

health of chickens and rats treated with bicarbonate-azide buffer versus those not dosed with the buffer have been observed in our laboratory. The volume of extract given was calculated by multiplying the body weight of the animal, the dose as percentage of body weight, and the concentration of extract. Treated chickens received daily doses equal to 0.8% of body weight and rats received 0.4% of body weight. These doses have been found to induce similar clinical signs and lesions observable at necropsy in the two species over a 3-day period (Marceau-Day, 1988).

D. Preparation of hepatic microsomes:

On the fourth day, the animals were weighed, sacrificed by guillotine, and examined for gross lesions. Livers were carefully removed, weighed, and placed on ice in 0.125 M KCl. Microsomes were prepared from the pooled livers of the group, treated or control. The livers were minced, rinsed, and homogenized in 4 volumes of ice cold buffer solution (10 mM tris, 0.25 M sucrose, and 1.0 mM disodium EDTA; pH 7.4). A microsomal pellet was prepared by stepwise differential centrifugation of the homogenate and its supernatant (Lake, 1987) at 600, 8000, and 14500 x g, and subsequent centrifugation of the supernatant at 105,000 x g. The resulting pellet was washed once with 0.125 M KCl and recentrifuged at 105,000 x g for 1 hr. All centrifugation steps were conducted at 4 C. The final microsomal pellet was

resuspended in 0.125 M KCL in 20% glycerol and frozen (-70 C) until used. Microsomes were used within two weeks of isolation.

E. Protein determination:

The concentration of microsomal protein was determined according to the method of Lowry et al. (1951). Microsomal suspensions (duplicates) were diluted 1:100 and 1:200 in 0.1 N NaOH. Protein standards (0, 30, 50, 100, and 200 $\mu\text{g/mL}$) were prepared from stock bovine serum albumin (Sigma fraction V, 10 mg/mL) in 0.1 N NaOH. To both sample and standard solutions (1mL) was added 5 mL of copper reagent (2% Na_2CO_3 , 0.4% NaOH, 0.025% Na_2CuEDTA) at 30 sec intervals followed by vortexing. Exactly 10 min later, 0.5 mL of freshly prepared Folin Ciocalteu reagent (diluted 1:1 in triply distilled water) was added and the solution was again vortexed. Following a 30 min incubation at room temperature, the absorbances were measured at 500 nm on a Bausch & Lomb Spectronic 2000 UV-visible spectrophotometer (Bausch & Lomb, Rochester, New York). The concentration of protein in the samples was determined from a standard curve constructed from the standards. A typical standard curve used in the protein determinations is shown in Appendix 1.

F. Cytochrome P-450:

The cytochrome P-450 content of the microsomes was determined by the method of Omura and Sato (1964b). Microsomes were diluted (1:10 for chickens and 1:20 for rats) in 0.1 M potassium phosphate buffer (pH 7.4). A few grains (approx. 0.1 g) of sodium dithionite was added, the solutions were mixed by inversion, and 1.0 mL aliquots were placed into two plastic cuvettes (10 mm path length). The cuvettes, reference and sample, were placed into the spectrophotometer and a baseline of zero absorbance between the two cuvettes was run from 300 to 500 nm and stored. The sample cuvette was then saturated with carbon monoxide (CO) by exposure to a stream of small bubbles for 30 sec. It was then returned to the spectrophotometer, and a difference spectrum was recorded between 350-500 nm. Depending upon the source of microsomes and the pretreatments the absorbance maximum were observed between 447-452 nm. The cytochrome P-450 content of the microsomes was determined from the difference in absorbance of the carbon monoxide complex of dithionite-reduced cytochrome P-450 and that of the non-liganded reduced cytochrome P-450. The change in absorbance at 450 nm relative to 490 nm was then converted to the specific content of cytochrome P-450 using an extinction coefficient of $91 \text{ cm}^{-1}\text{mM}^{-1}$ (Omura and Sato, 1964b). The final concentration was expressed as nmole cytochrome P-450 per mg of microsomal protein.

G. Cytochrome b_5 :

The concentration of cytochrome b_5 in the microsomal suspensions was determined by the method of Omura and Sato (1964b). Microsomes were diluted (1:10 for chickens and 1:20 for rats) in 0.1 M potassium phosphate buffer (Ph 7.4) and 1.0 Ml aliquots were placed into two plastic cuvettes. The cuvettes, reference and sample, were placed into the spectrophotometer and a baseline of zero absorbance was run between the sample and reference cuvettes in the range of 300-500 nm and stored. The cuvettes were removed and 5 μ L of a freshly prepared NADH solution (30 Mm) was added to the sample cuvette and 5 μ L of water was added to the reference cuvette. The cuvettes were inverted to mix, returned to the spectrophotometer, and scanned from 350-490 nm.

The addition of NADH to the contents of the sample cuvette resulted in the rapid reduction of cytochrome b_5 as characterized by an increase in absorbance at about 556 nm and 426 nm and a loss of absorbance at about 409 nm. The microsomal concentration of cytochrome b_5 was determined by applying the millimolar difference extinction coefficient of 185 $\text{mM}^{-1}\text{cm}^{-1}$ for the difference in absorbance between 426 and 409 nm. The cytochrome b_5 content was expressed as nmoles cytochrome b_5 per mg of microsomal protein.

H. NADH- and NADPH-dependent cytochrome c reductase:

NADH- and NADPH-dependent cytochrome c reductase activities were assayed by use of the method of Phillips and Langdon (1962) as modified by Yasukochi and Masters (1976). Microsomal suspensions were diluted 1:10 in 0.125 M KCL. A working stock solution of cytochrome c was prepared by solubilizing 62 mg of cytochrome c (Type III, from horse heart) in 10 mL of water and 34 mL potassium phosphate buffer (1 M, pH 7.7) and then diluting to 100 mL with triply distilled water. Sample and reference solutions were prepared by pipetting 2.5 mL of cytochrome c solution (room temperature) followed by 5 μ L of the microsomal dilution into two 1 cm plastic cuvettes. The spectrophotometer was balanced between the two cuvettes and the parameters were set for absorbance measurements every 30 sec for 180 sec at 550 nm using the built-in kinetic program. The reaction was initiated by the addition of 10 μ L of freshly prepared NADH (10mM) or NADPH (10mM) to the sample cuvette. The sample cuvette was inverted to mix and returned to the sample chamber. The reductase activities were quantified using an extinction coefficient of $29.5 \text{ mM}^{-1}\text{cm}^{-1}$ for reduced cytochrome c. The final NADH- and NADPH-cytochrome c reductase activities were expressed as nmol of cytochrome c reduced per min per mg of microsomal protein.

I. Hepatic microsomal monooxygenase activities:

1. Aminopyrine N-demethylase:

The microsomal-mediated metabolism of aminopyrine was measured by determination of the product, formaldehyde. The reaction mixture consisted of 1.5 mg of microsomal protein, 0.3 mM NADP⁺, 10 mM glucose 6-phosphate, 7 units glucose 6-phosphate dehydrogenase, 10 mM MgCl₂, 100 mM potassium phosphate buffer (pH 7.4), and 10 mM aminopyrine in a final volume of 3.0 mL. The reaction was started by the addition of the NADPH-generating system (glucose 6-phosphate and glucose 6-phosphate dehydrogenase), incubated at 37 C for 15 min, and stopped by the addition of 1 mL 70% trichloroacetic acid. Aminopyrine N-demethylase (APND) was quantified by measuring the amount of formaldehyde formed according to the method of Nash (1953) as compared with a standard curve prepared in the reaction matrix. A typical standard curve used in the determination of activity is shown in Appendix 2. Aminopyrine N-demethylase activity was expressed as nmoles of formaldehyde formed per min per mg microsomal protein and nmoles of formaldehyde formed per min per nmole cytochrome P-450.

2. Aniline hydroxylase:

Aniline hydroxylase activity (ANH) was measured by following the formation of p-aminophenol from aniline according to the method of Lake (1987) with modifications. The 2 mL reaction mixture contained 1 mg of microsomal protein, 50 mM Tris-HCL

buffer (pH 7.8), 5 mM MgSO_4 , 0.5 mM NADP^+ , 5 mM glucose 6-phosphate, 1.5 units of glucose 6-phosphate dehydrogenase, and 5 mM aniline-HCl. The reaction was started by the addition of the NADPH-generating system (glucose 6-phosphate and glucose 6-phosphate dehydrogenase) and was incubated in a 37 C water bath for 10 min. The reaction was terminated by adding 2 mL of 10% trichloroacetic acid. After centrifugation, a 2 mL aliquot of the supernatant was added to 1 mL of 10% Na_2CO_3 followed by the addition of 2 mL of 1% phenol in 0.1 N NaOH. The resulting optical density was measured at 620 nm after 30 min and compared with a p-aminophenol standard. A typical p-aminophenol standard curve is shown in Appendix 3. Aniline hydroxylase activity was expressed as nmoles of p-aminophenol formed per min per mg of microsomal protein and nmoles of p-aminophenol formed per min per nmole of cytochrome P-450.

3. Aldrin epoxidase:

Aldrin epoxidase activity (AE) was determined from the amount of dieldrin produced as described by Riviere et al. (1985) with modifications. The incubation system contained 50 mM potassium phosphate buffer (pH 6.8); 0.25 mM NADP^+ ; 2.5 mM glucose-6-phosphate; 1 unit glucose-6-phosphate dehydrogenase; 0.2 mg of microsomal protein; and 50 μM aldrin in a final volume of 1.0 mL. Incubation was performed aerobically in a constant temperature shaker at 37 C for 15 min. The reaction was initiated by the addition of aldrin and stopped by the

addition of 0.2 mL of acetone. Hexane (5 mL) was added and the tubes were capped and vortexed for 45 sec. This resulted in extraction of dieldrin from the incubation mixture (bottom layer) into the hexane layer (top layer). Dieldrin was assayed by use of a Hewlett Packard 5880A gas chromatograph (Hewlett Packard, Avondale, PA) equipped with an electron-capture detector (nickel-63) and a capillary column (15 m x, 0.53 mm ID, phase 007-17 Quadrex Corp., New Haven, CT). The column, detector, and injector temperatures were 220-240 C (10 C per min), 300 C, and 180 C, respectively. The injection of samples was made in the splitless mode using a direct flash injection liner. The carrier gas was 5% methane in argon at a flow rate of 11 mL per minute. Carrier gas was also used for detector make-up to a total flow of ca. 30 mL per minute. The amount of dieldrin in the extract was quantified by peak height from a standard curve. A typical standard curve used in the determination of aldrin epoxidase activity is shown in Appendix 4. The aldrin epoxidase activity was expressed as nmoles of dieldrin produced per min per mg of microsomal protein and nmoles of dieldrin produced per min per nmole of cytochrome P-450.

4. Ethoxycoumarin O-deethylase:

The O-dealkylation of 7-ethoxycoumarin was measured fluorometrically using a spectrophotofluorometer (Farrand Optical Co., Valhalla, NY) by the method of Lake (1987). The

ethoxycoumarin O-deethylase (ECOD) assay was run aerobically at 37 C for 10 min and contained a standard reaction mixture of 50 mM Tris-HCl buffer (pH 7.8); 5 mM MgSO_4 ; 0.5 mM NADP⁺; 5 mM glucose-6-phosphate; 3 units of glucose-6-phosphate dehydrogenase; 0.5 mM 7-ethoxycoumarin and 1.0 mg of microsomal protein in a final volume of 2.0 mL. The reaction was started with the addition of substrate and terminated by the addition of 1.0 mL of zinc sulfate solution (5% w/v) followed by 1.0 mL of saturated barium hydroxide to all tubes. After centrifugation, 1.5 mL of supernatant was removed and added to 3.0 mL of 0.5 M glycine-sodium hydroxide buffer (pH 10.5). The ECOD assay was measured using 380 nm excitation and 452 nm emission wavelengths and was compared with an 7-hydroxycoumarin (umbelliferone) standard. A typical 7-hydroxycoumarin standard curve is shown in Appendix 5. The ECOD activity was expressed as nmoles 7-hydroxycoumarin formed per min per mg of microsomal protein and nmoles 7-hydroxycoumarin formed per min per nmole of cytochrome P-450.

5. Ethoxyresorufin O-deethylase activity:

The O-dealkylation of 7-ethoxyresorufin was also measured fluorometrically by the method of Lake (1987). The 7-ethoxyresorufin O-deethylase (EROD) assay was nearly identical to the ECOD assay. The EROD assay was also performed aerobically at 37 C for 10 min and contained the components of the ECOD reaction mixture with the exception that 5 μM 7-

ethoxyresorufin was used for the substrate and the microsomal protein concentration was 0.1 mg per mL. The pH of the glycine-sodium hydroxide buffer that was added to the supernatant following centrifugation was raised to 8.5 for EROD. The 7-ethoxyresorufin O-deethylase activity was measured using 535 nm excitation and 582 nm emission wavelengths and quantitated with resorufin as a standard. A typical resorufin standard curve is shown in Appendix 6. The EROD activity was expressed both as pmoles of resorufin formed per min per mg of microsomal protein and pmoles of resorufin formed per min per nmole of cytochrome P-450.

6. Aryl hydrocarbon hydroxylase:

Aryl hydrocarbon hydroxylase activity was measured by the method described by Guengerich (1982). The components of a 1.0 mL reaction mixture included: 50 mM potassium phosphate buffer (pH 7.4), 5.0 mM $MgCl_2$, 1.0 mM NADP⁺, 10.0 mM glucose 6-phosphate, 3 units glucose 6-phosphate dehydrogenase, 80 μ M benzo(a)pyrene, and 0.1 mg of microsomal protein. The reaction was started with the NADPH-generating system (glucose 6-phosphate and glucose 6-phosphate dehydrogenase). Incubations were performed aerobically at 37 C for 10 min and were terminated with 1.0 mL of cold acetone (4 C). The products were extracted with 3.25 mL hexane followed by the addition of 1 N NaOH. Fluorescence of the aqueous layer was read using an excitation wavelength of 396 nm and an emission

wavelength of 522 nm. Aryl hydrocarbon hydroxylase activity was quantitated using a 3-OH benzo(a)pyrene standard. A typical 3-OH benzo(a)pyrene standard curve is shown in Appendix 7. Activities were expressed as pmoles of 3-OH benzo(a)pyrene formed per min per mg of microsomal protein and the pmoles of 3-OH benzo(a)pyrene formed per min per nmole of cytochrome P-450.

J. Statistical analysis:

The significance of difference between nontreated controls and treated groups was determined using an unpaired Student's t test. The acceptable level of significance for this study was established at $p < 0.05$.

RESULTS:

Body weights and liver weights:

Decreases in body weights in chickens were detected in the S. drummondii-treated groups, compared with controls. These weight losses developed despite the finding of large quantities of food material in the crops of treated chickens at necropsy. The treated rats had slight, but not significant, decreases in gains in body weight relative to controls. Liver weight changes paralleled those of body weight in the treated animals, resulting in liver to body weight ratios similar to those of the control animals (Table 1).

Hepatic microsomal cytochromes and reductases:

Marked differences in content of microsomal cytochrome P-450 of S. drummondii-treated vs control animals were detected (Table 2). An approximate 2-fold increase in chickens' and a 2-fold decrease in rats' cytochrome P-450 specific content were consistently observed for the treated groups. Significant differences were not observed in the cytochrome b₅ content of microsomes from S. drummondii-treated chickens or rats.

The effect of S. drummondii treatment on NADPH- and NADH-cytochrome c reductase activities followed a pattern similar to that described for the cytochromes. A 2.5-fold increase

Table 1. Effect of Sesbania drummondii treatment on body and liver weights in chickens and rats

	Chickens		Rats	
	Control	Treated	Control	Treated
Body weight (% change)*	+7.2 \pm 2.3	-8.1 \pm 3.0†	+5.6 \pm 3.4	+1.8 \pm 6.8
Relative liver/body weight (% body weight)†	+2.0 \pm 0.1	+2.0 \pm 0.1	+3.9 \pm 0.3	+3.8 \pm 0.3

Data are expressed as group means (\pm SEM), n = 3).

* The results were calculated according to this formula: $\frac{\text{Final weight} - \text{First day weight}}{\text{First day weight}} \times 100$

† The results were calculated according to this formula: $\frac{\text{Pooled liver weight}}{\text{Pooled body weight}} \times 100$

‡ Significantly different (Student's t test) from control data (P<0.01).

Table 2. Effect of *Sesbania drummondii* treatment on hepatic microsomal monooxygenase cytochromes and reductases in chickens and rats

	Chickens		Rats	
	Control	Treated	Control	Treated
Cytochrome P-450 (nmol/mg protein)	0.17 \pm 0.04	0.36 \pm 0.02†	0.71 \pm 0.06	0.43 \pm 0.09*
Cytochrome b ₅ (nmol/mg protein)	0.11 \pm 0.04	0.18 \pm 0.06	0.21 \pm 0.04	0.22 \pm 0.07
NADH cytochrome c reductase (nmol reduced/min/mg protein)	160 \pm 35	178 \pm 26	607 \pm 16	525 \pm 102
NADPH cytochrome c reductase (nmol reduced/min/mg protein)	64 \pm 16	156 \pm 12†	99 \pm 28	66 \pm 29

Data are expressed as group means (\pm SEM), n = 3. Significantly different (Student's *t* test) from control data (* *P*<0.05, †*P*<0.01).

in the chickens and slight, but not significant, decrease in the rats were observed for NADPH-cytochrome c reductase activity. Significant differences were not observed for NADH-cytochrome c reductase activity in either species treated with S. drummondii.

Hepatic microsomal monooxygenase activities:

The effects of S. drummondii-treatment on six selected hepatic microsomal monooxygenase enzyme activities were compared in terms of mg of microsomal protein (specific activity) and nmoles of cytochrome P-450 (molar activity or turnover number; Table 3).

When expressed per mg of microsomal protein, increases in all six activities were evident with the treated chickens. Two-fold increases were detected for aminopyrine N-demethylase, aniline hydroxylase, ethoxycoumarin O-deethylase, and arylhydrocarbon hydroxylase activities. Aldrin epoxidase activity was increased 4-fold in treated birds. The most pronounced change was observed for ethoxyresorufin O-deethylase activity, with a 15-fold increase in treated birds, compared with controls.

Significant decreases in three activities, expressed per mg of microsomal protein, were observed in the treated rats. A 40% reduction in aminopyrine N-demethylase, aniline

Table 3. Effect of *Sesbania drummondii* treatment on selected hepatic microsomal monooxygenase activities.

	Chickens		Rats	
	Control	Treated	Control	Treated
Aminopyrine-N-demethylase (nmoles HCHO/min/mg protein) (nmoles HCHO/min/nmole P-450)	3.09±1.05 18.00±3.48	6.19±1.52* 17.46±4.90	3.38±0.18 4.78±0.61	2.10±0.20† 5.04±1.56
Aniline hydroxylase (nmoles p-aminophenol/min/mg protein) (nmoles p-aminophenol/min/nmole P-450)	2.38±0.55 14.01±1.32	5.68±1.26* 16.09±4.28	1.96±0.07 2.76±0.35	1.11±0.05† 2.66±0.64
Aldrin epoxidase (nmoles dieldrin/min/mg protein) (nmoles dieldrin/min/nmole P-450)	0.61±0.15 3.63±0.99	2.65±0.48† 7.49±1.72*	1.56±0.56 2.24±0.99	0.92±0.21 2.14±0.33
7-Ethoxycoumarin-O-deethylase (nmoles 7-OH coumarin/min/mg protein) (nmoles 7-OH coumarin/min/nmole P-450)	3.89±0.45 23.19±2.27	7.68±0.99† 21.62±3.42	3.99±0.18 5.64±0.76	2.39±0.12† 5.70±1.37
7-Ethoxyresorufin-O-deethylase (pmoles resorufin/min/mg protein) (pmoles resorufin/min/nmole P-450)	8±1 49±14	123±18† 344±51†	279±102 401±181	89±6 210±31
Aryl hydrocarbon hydroxylase (pmole 3-OH benzo()pyrene/min/mg protein) (pmole 3-OH benzo()pyrene/min/nmole P-450)	175±48 1024±70	423±17† 1187±60*	214±40 305±80	128±8 308±86

Data are expressed as group means (± SEM), n = 3. Significantly different (Student's *t* test) from control data (**P*<0.05, †*P*<0.01)

hydroxylase, and ethoxycoumarin O-deethylase activities developed in the treated rats. Statistically insignificant decreases in aldrin epoxidase, arylhydrocarbon hydroxylase, and ethoxyresorufin O-deethylase were observed in treated rats.

More specific changes in enzyme activities were evident for activities expressed as the turnover number for the cytochrome(s) P-450 (units per nmole of P-450). Only arylhydrocarbon hydroxylase, aldrin epoxidase, and ethoxyresorufin O-deethylase activities were increased in the treated chickens approximately 1.2-fold, two-fold, and seven-fold, respectively. Significant decreases in enzyme activities were not detected in rats.

DISCUSSION:

This study indicates that extracts of Sesbania drummondii markedly affect the hepatic microsomal monooxygenase systems of chickens and rats. In White Leghorn chickens, S. drummondii treatment resulted in induction of the hepatic microsomal monooxygenase system. This finding was shown by the increases in cytochrome P-450 content, NADPH-cytochrome c reductase activity, and the activities of six cytochrome P-450 mediated enzyme activities.

The consequence of induction of the cytochrome P-450 monooxygenase system in the chicken by Sesbania drummondii on the outcome of chemical biotransformation of both endogenous and exogenous substrates is related to the changes induced in the relative proportions of the various cytochrome P-450 isozyme families. Individual cytochrome P-450 isozymes exhibit variable substrate and reaction specificity. Therefore, changes in the proportions of various forms of cytochrome P-450 isozymes may determine which reactions predominate and whether detoxication or bioactivation will result.

Specific changes in the cytochrome P-450 isozymal distribution pattern have been related to exposure to particular chemicals. Goldstein (1980) described a classification scheme for chemical inducers consisting of three types: phenobarbital, 3-

methylcholanthrene, and mixed. Much work has been performed in mammals to characterize the effects of phenobarbital, 3-methylcholanthrene, and mixed- types of induction on the cytochrome P-450 monooxygenase system and on xenobiotic biotransformation. Therefore, it is convenient to compare the effects of new chemicals on the components of the cytochrome P-450 monooxygenase system with the effects induced by the three classical chemical inducers.

The cytochrome P-450 content of chicken hepatic microsomes is increased by all three classical chemical inducers. Phenobarbital increased the cytochrome P-450 content by 3 to 4-fold in White Leghorn chickens (Strittmatter and Umberger, 1969; Powis et al., 1976; Darby et al., 1985; Lorr and Bloom, 1987). Inductions of cytochrome P-450 specific content from 3 to 6-fold have been reported with the 3-methylcholanthrene-type inducers; 3-methylcholanthrene, β -naphthoflavone, and 2,3,7,8 tetrachlorodibenzo-p-dioxin (Buckpitt and Boyd, 1982; Ehrich and Larsen, 1983; Sawyer et al., 1986; Darby et al., 1985). Arochlor 1254, a mixed-type of inducer, increased cytochrome P-450 specific content 6-fold in White Leghorn chicks (Rinzky and Perry, 1983). Additionally, a number of other compounds (non-classical) have been reported to increase chicken cytochrome P-450 content. Variable increases (2 to 4-fold) were reported with prochloraz (Riviere et al., 1985), S,S,S-tri-*n*-butyl phosphorotrithioate (Lapadula et al., 1984),

2-acetylaminofluorene (Darby et al., 1985) and DDT (Sell et al., 1971).

Sesbania drummondii treatment of White Leghorn chickens increased cytochrome P-450 specific content by 2-fold. The magnitude of this increase was lower than reported with phenobarbital, 3-methylcholanthrene, and mixed induction of chickens. By comparison, similar (2-fold) increases were observed with treatment of non-classical inducers.

Increased NADPH-cytochrome c reductase activity in mammals is associated with phenobarbital-type of induction (Snyder and Remmer, 1979). In chickens, slight to moderate increases in NADPH-cytochrome c reductase activity have been observed with phenobarbital, mixed, and non-classical inducers. Pilch and Combs (1981) reported a moderate increase (approx. 2-fold) in reductase activity in 12-day old cockerels given phenobarbital and fed diets supplemented with vitamin E and selenium. This increase was less pronounced without vitamin E and selenium supplements in male chicks and in female chicks with or without the supplements. Arochlor 1254 slightly elevated (1.5-fold) NADPH-cytochrome c reductase activity in White Leghorn chickens (Rinzky and Perry, 1983). Sonali pearl chicks treated with caffeine for 5 days also had slight increases (1.5-fold) in NADPH-cytochrome c reductase activity (Govindwar et al., 1984). No change in NADPH-cytochrome c

reductase activity were reported with 3-methylcholanthrene (Buynitzky et al., 1978), phenolic compounds (Klasing et al., 1985), estradiol implants (Takahashi and Jensen, 1984) or dietary changes (Brenes et al., 1985).

The effect of Sesbania drummondii treatment on NADPH-cytochrome c reductase activity resembled the increases observed with phenobarbital, Arochlor 1254, and caffeine. However, the magnitude of the increase (nearly 2.5-fold) was greatest with Sesbania drummondii. It is interesting to note that in Sprague-Dawley rats a similar increase (2.8-fold) in NADPH-cytochrome c reductase activity was reported with pregnenolone-16 α -carbonitrile (Gorski et al., 1985).

Changes in cytochrome P-450-mediated enzyme activities in the chicken are dependent on the chemical inducer as well as the specific enzyme activity. Increases in aminopyrine N-demethylase activity in chicken hepatic microsomes are reported with phenobarbital and mixed-type of inducers. Moderate (2-fold) increases in this activity in chickens were reported with phenobarbital (Pilch and Combs, 1981; Darby et al., 1985; Lorr and Bloom, 1987) and polychlorinated biphenyls (Rinzky and Perry, 1983; Cecil et al., 1978; Rifkind et al., 1984).

Aldrin epoxidase activity of chicken hepatic microsomes was

increased 4-fold with Arochlor (Rinzky and Perry, 1983) and 2.5-fold with prochloraz (Riviere et al., 1985), but was decreased 2-fold with 2,3,7,8-tetrachlorodibenzo-p-dioxin (Sawyer et al., 1986). To the best of our knowledge, the effect of phenobarbital induction on aldrin epoxidase activity in chickens has not been reported.

Aniline hydroxylase activity was increased in chickens treated with phenobarbital, 3-methylcholanthrene, mixed, and other chemical inducers. Aniline hydroxylase activities increased 2-fold with phenobarbital (Pilch and Combs, 1981), 3-methylcholanthrene (Buynitzky et al., 1978) and Arochlor (Cecil et al., 1978). Larger increases of 4-fold were induced by prochloraz (Riviere et al., 1985) and S,S,S-tri-*n*-butyl phosphorotriithioate (Lapadula et al., 1984).

The effect of the classical chemical inducers on ethoxycoumarin O-deethylase activity in chickens has been limited to the mixed-type. A 2-fold increase in activity was reported in chicken embryo livers with 4 polychlorinated biphenyl congeners (Rifkind et al., 1984). No change in ethoxycoumarin O-deethylase activity was observed with phenobarbital-type (Rifkind et al., 1982) or 3-methylcholanthrene-type (Darby et al., 1986) inducers in chickens.

Induction of ethoxyresorufin O-deethylase in chickens has been reported with 3-methylcholanthrene, mixed, and non-classical chemical inducers. Marked increases of 40-fold with 2,3,7,8-tetrachlorodibenzo-p-dioxin (Sawyer et al., 1986) and 50-fold with polychlorinated biphenyl congeners (Rifkind et al., 1984) were observed in ethoxyresorufin O-deethylase activity. A much smaller increase of 3-fold was detected with prochloraz treatment of chickens (Riviere et al., 1985).

Arylhydrocarbon hydroxylase activity was induced in chickens treated with all 3 classical and other non-classical inducers. Darby et al. (1985) reported small increases of 1.5-fold with phenobarbital and 2-fold with 2-acetylaminofluorene in chicken hepatic microsomal arylhydrocarbon hydroxylase activity. Larger increases of 8 to 20-fold of arylhydrocarbon hydroxylase were reported with treatments of polychlorinated biphenyl congeners (Rifkind et al., 1984) and 3-methylcholanthrene (Buynitzky et al., 1978).

The Sesbania drummondii-induced changes in the chicken hepatic microsomal cytochrome P-450-mediated enzyme activities did not parallel any of the three classical inducers through all six of the enzyme activities. With some of the activities, Sesbania drummondii treatment resembled phenobarbital induction, but with other activities it resembled 3-methylcholanthrene or mixed induction. Sesbania drummondii

treatment induced essentially 2-fold increases in aminopyrine N-demethylase, aniline hydroxylase, arylhydrocarbon hydroxylase, and ethoxycoumarin O-deethylase. Similar increases in these activities in chickens were induced with phenobarbital and polychlorinated biphenyls. The 4-fold induction of aldrin epoxidation with Sesbania drummondii treatment was of a similar magnitude as reported with Arochlor 1254. Sesbania drummondii induced ethoxyresorufin O-deethylase activity as did 2,3,7,8-tetrachlorodibenzo-p-dioxin and polychlorinated biphenyls. However, the Sesbania drummondii induced increase was 15-fold, much less than the 40-fold increase with 2,3,7,8-tetrachlorodibenzo-p-dioxin and 50-fold increase with the polychlorinated biphenyls.

The overall response profile of the hepatic monooxygenase system (cytochromes, reductases, enzyme activities) of White Leghorn chickens to Sesbania drummondii was somewhat different from that reported for the classical inducers. Sesbania drummondii is a complex mixture of compounds, and in the chicken, may be a new unique type of inducer. However, further studies will be necessary to confirm this.

Usually compounds that are inducers of the microsomal monooxygenase system in avian species are also inducers of the monooxygenase systems in mammals. However, this was not the case with Sesbania drummondii. A depression, rather than

induction, of cytochrome P-450 content and all the activities of the hepatic monooxygenase system was observed in microsomes from the S. drummondii-treated rat.

Although the specific activities (nmole of product per min per mg of microsomal protein) for all the substrates catalyzed by microsomes from S. drummondii-treated chickens were higher than those for controls, the molar activities (nmoles product per min per nmole cytochrome P-450) were essentially the same except in the case of aldrin epoxidase and ethoxyresorufin O-deethylase, which were 2-fold and 7-fold higher, respectively, with microsomes from the Sesbania drummondii-treated chickens, compared with microsomes from control chickens. These data suggest induction of at least 1 new isozyme in the chicken liver. In rats, S. drummondii appeared to suppress the constitutive isozymes and, in the case of ethoxyresorufin O-deethylase induce a novel isozyme of lower activity. More rigorous proof remains to be established. At the present time it is not known whether the same component in Sesbania drummondii produces the effects seen in chickens and rats. The mechanisms for monooxygenase induction of chickens and depression in rats by S. drummondii are not presently known. Whether similar responses will occur in other avian and mammalian species will require further study.

EXPERIMENT 2 - Evaluation of the effects of the bicarbonate-azide extraction buffer/vehicle on the hepatic cytochrome P-450 monooxygenase systems of chickens and rats.

OBJECTIVES:

1. To compare the effect of treatment with the bicarbonate-azide extraction buffer/vehicle vs. no treatment on hepatic microsomal cytochrome P-450 specific content in chickens and rats.
2. To compare the effect of treatment with bicarbonate-azide extraction buffer/vehicle vs. no treatment on two selected hepatic cytochrome P-450 mediated enzyme activities: aminopyrine N-demethylase and ethoxyresorufin O-deethylase.

MATERIALS AND METHODS:

A. Chemicals:

The chemicals and biochemicals were as described in detail in Experiment 1.

B. Animals:

Four male and female 250- to 350-g White Leghorn chickens were group housed in a commercial brooder. Six male 300- to 400-g Sprague Dawley rats were maintained 3 per cage on a 12-hr light/dark cycle in a temperature (21-24 C) and humidity

(40-60%) controlled environment. Chickens and rats received feed (Purina chick Startena and Purina rat chow) and water ad libitum. Both species were acclimated for one week before the study began. The chicken experiment was performed with one group of 2 treated and 2 control animals. The rat experiment was performed with one group of 3 treated and 3 control animals.

C. Treatments:

The treated animals received three daily gavages of the sodium bicarbonate-sodium azide buffer (BBN) (25mM sodium bicarbonate, pH 7.4; 0.02% sodium azide). The control animals were not given the extracts. The volume of extract administered was calculated by multiplying the body weight of the animal and the dose as percentage of body weight. The treated chickens received daily doses equal to 0.8% of body weights and the treated rats received 0.4% of body weights.

D. Preparation of hepatic microsomes:

Microsomes were prepared from the pooled livers of the group, treated or control, as described in detail in Experiment 1.

E. Protein determination:

The concentration of protein in the microsomal suspensions was determined as described in detail in Experiment 1.

F. Cytochrome P-450:

The concentration of cytochrome P-450 of the microsomes was determined as described in detail in Experiment 1.

G. Hepatic microsomal monooxygenase activities:

1. Aminopyrine N-demethylase:

The aminopyrine N-demethylase activity of the microsomal suspensions was measured as described in detail in Experiment 1.

2. Ethoxyresorufin O-deethylase:

The O-dealkylation of 7-ethoxyresorufin was measured as previously described in detail in Experiment 1.

RESULTS:

The cytochrome P-450 content and hepatic microsomal monooxygenase activities of chickens and rats treated with the bicarbonate-azide buffer vs. those animals not treated with the buffer were essentially the same (Table 4). BBN treated-chickens had only slightly higher cytochrome P-450 content and ethoxyresorufin O-deethylase activity and slightly lower aminopyrine N-demethylase activity than the untreated controls. The BBN-treated rats showed slightly lower cytochrome P-450 content but virtually identical aminopyrine N-demethylase and ethoxyresorufin O-deethylase activities to the control rats.

Table 4: The effect of bicarbonate-azide buffer (BBN) vehicle on cytochrome P-450 content and hepatic microsomal monooxygenase activities in chickens and rats

	Chickens		Rats	
	Control	BBN	Control	BBN
Cytochrome P-450 (nmole/mg protein)	0.19	0.21	0.61	0.55
Aminopyrine N-demethylase (nmoles HCHO/min /mg protein)	1.33	1.19	1.40	1.41
Ethoxyresorufin O- deethylase (pmoles resorufin/min/ mg protein)	47	61	201	199

Values are the mean of duplicate analyses per group.

DISCUSSION:

Azide is a known potent inhibitor of heme-iron containing enzymes such as catalase, peroxidase, and cytochrome oxidase (Keilin, 1936-1937; Smith et al., 1977; Winston and Cederbaum, 1982). Therefore, it was of interest to determine whether the oral intake of azide with bicarbonate buffer altered the hepatic microsomal cytochrome P-450 monooxygenase system. Results of this study indicate that the bicarbonate-azide buffer when administered orally does not induce changes in the cytochrome P-450 monooxygenase system of chickens and rats. This finding was likely due to the relatively small amount of azide administered to our experimental animals. Our chickens and rats received approximately 5 mL per day of a 0.02% solution of sodium azide which was equivalent to a dosage of 1 mg of sodium azide per day or 3 mg of sodium azide per kg of body weight. In comparison, the acute lethal dosage of oral sodium azide in the rat is 27 mg per kg of body weight (Gosselin et al., 1984). This study was performed to support the results of Experiment 1 in which treated animals were gavaged with a solution consisting of an extract of S. drummondii in the BBN buffer and the control animals did not receive the extract nor the buffer. This also supports the use of BBN buffer in our studies of S. drummondii-induced effects in chickens and rats. Subsequent experiments utilized control animals treated with the BBN buffer rather than nontreated controls.

EXPERIMENT 3 - Comparison of the effects of an extract of Sesbania drummondii on the hepatic cytochrome P-450 monooxygenase systems of male versus female rats.

OBJECTIVES:

1. To determine if the S. drummondii-induced effects on the hepatic cytochrome P-450 monooxygenase system in rats is altered by the sex of the animal.

MATERIALS AND METHODS:

A. Chemicals:

The chemicals and biochemicals were as described in detail in Experiment 1.

B. Animals:

Eight adult male (300-400 g) and eight adult female (250-300 g) Sprague-Dawley rats were housed in groups of 4 in wire mesh bottom cages. Food and water were available ad libitum. The room where the rats were kept was environmentally controlled at 21-24 C with a 12 hr light/dark cycle. The rats were acclimated for 7 days prior to their random assignment to control and treatment groups. The study was performed with 2 groups of 4 animals, 2 treated and 2 control in each group, for both male and female rats.

C. Treatments:

Extracts of S. drummondii were prepared as described in detail in Experiment 1. The treated groups were weighed daily for 3 consecutive days and then gavaged with the extract of S. drummondii. Control groups were also weighed daily for 3 days and were given equivalent volumes of the BBN extraction buffer (without S. drummondii). The volume of extract given was calculated by multiplying the body weight of the rat, the dose as percentage of body weight, and the concentration of the extract. Treated and control rats received daily doses equal to 0.5% of body weight.

D. Preparation of hepatic microsomes:

Microsomes were prepared from the pooled livers of the group, treated or control, as described in detail in Experiment 1.

E. Protein determination:

The concentration of microsomal protein was determined as described in detail in Experiment 1.

F. Cytochrome P-450:

The cytochrome P-450 content of the microsomal suspensions was measured as described in detail in Experiment 1.

RESULTS:

The cytochrome P-450 content of S. drummondii-treated male and female rats is shown in Table 5. Decreases in the cytochrome P-450 content were observed in the microsomes of both S. drummondii-treated male and female rats. The magnitude of reduction in the cytochrome P-450 content was slightly greater in the treated-male rats than the treated-female rats. Male S. drummondii-treated rats had an approximate 40% reduction whereas the treated females had an approximately 30% reduction in cytochrome P-450 content. Control female rats were also noted to have lower cytochrome P-450 contents than their male counterparts.

Table 5: Cytochrome P-450 content of Sesbania drummondii-treated male and female rats

Sex	Cytochrome P-450*	
	Control	Treated
Male	0.96	0.56
Female	0.75	0.53

* Cytochrome P-450 expressed as nmole cytochrome P-450/mg protein. Values are the means, n = 2.

DISCUSSION:

Sex-related differences in the hepatic biotransformation of xenobiotics are well established in the rat (Conney, 1967; Kato, 1974; Gustafsson and Stenberg, 1974). These differences result in variable pharmacologic and toxicologic responses to a number of xenobiotics. In general, male rats exhibit higher rates of metabolic conversion of a number of substrates including hexobarbital, aminopyrine and ethylmorphine than do females (Conney, 1967; Kato, 1974; Gustafsson and Stenberg, 1974). As a result, xenobiotics may be more rapidly detoxicated or bioactivated in the male rat. An example of clinical toxicosis related to the capability of male rats to convert chemicals to reactive intermediates faster than females is observed with carbon tetrachloride. Male rats are more susceptible to liver injury produced by carbon tetrachloride than are females (Sipes and Gandolfi, 1986).

Some of the sex-related variances in xenobiotic biotransformation are due to differences in their hepatic cytochrome P-450 monooxygenase systems. Differences between male and female rats have been observed in cytochrome P-450 specific content, NADPH-cytochrome P-450 reductase, and cytochrome P-450-mediated activities (Sipes & Gandolfi, 1986; Chengelis, 1988). In general, male rat hepatic microsomes contain 20 to 30% more of the above components. Our results concur with this finding. We observed control male Sprague-

Dawley rats to have 22% higher cytochrome P-450 content than their female counterparts.

Sex-related variances in xenobiotic biotransformation by the hepatic cytochrome P-450 monooxygenase system reflect differences in the profile of cytochrome P-450 isozymes in the liver microsomes (Sipes and Gandolfi, 1986). Cytochrome P-450 forms specific for both male (P-450h) and female (P-450i) rats have been identified (Sipes and Gandolfi, 1986). The balance between male and female sex hormones contribute to the relative properties of the cytochrome P-450 isozyme forms (Sipes and Gandolfi, 1986). Expression of cytochrome P-450h is dependent on testosterone and subject to neonatal imprinting by androgens which may be mediated through the male-specific pattern of growth hormone release (Ryan and Levin, 1990). Female rats do not have detectable amounts of cytochrome P-450h, even after removal of the ovaries, unless testosterone was administered in conjunction with ovariectomy (Waxman et al., 1985). However, a recent report questions the role of androgens. Bitar and Shapiro (1987) recently found that androgenic regulation of the hepatic monooxygenase system occurs only at suprapharmacologic levels and that females were in fact unresponsive to physiological levels of testosterone.

Not all animal species have demonstrated sex-related differences in hepatic biotransformation. Despite the large

sex variations observed in rats and certain strains of mice, these variations are uncommon in other species (Sipes and Gandolfi, 1986). Male and female birds of the same species are seldom very different (Ronis and Walker, 1989).

The S. drummondii-induced depression of cytochrome P-450 in adult rats was not altered by the sex of the animal. A reduction of cytochrome P-450 content was observed in both male and female rats. From this limited study, it appears that the male is slightly more sensitive to S. drummondii-induced depression than the female. However, additional experiments utilizing more groups of animals are required before statements of significance may be made.

EXPERIMENT 4 - Evaluation of the effects of an extract of Sesbania drummondii on microsomal membrane stability.

OBJECTIVES:

1. To determine if the Sesbania drummondii-induced effects on the hepatic cytochrome P-450 monooxygenase system are the result of changes in microsomal membrane stability as measured by glucose-6-phosphatase activity.

MATERIALS AND METHODS:

A. Chemicals:

The ammonium salt of molybdic acid, disodium salt of EDTA, the Fiske and Subbarow reducer (1-amino-2-naphthol-4-sulfonic acid, 0.8%, sodium sulfite and sodium bisulfite), monosodium salt of D-glucose-6-phosphate and L-histidine were obtained from Sigma Chemical Co. (St. Louis, MO). Dibasic sodium phosphate and trichloroacetic acid were purchased from Mallinckrodt, Inc. (Paris, KY). These chemicals and biochemicals were of analytical grade or better.

B. Animals:

One day-old White Leghorn chickens were obtained from the Department of Veterinary Science, Louisiana State University, Baton Rouge, LA. At 4 weeks of age, the birds were sexed and

the male chickens were separated for use in experimental studies. Fifty-four of the male chickens (200-400 g) were group housed , 18 per cage, in commercial brooders. Twenty-four male Sprague-Dawley rats (300-500 g) were obtained from Hilltop Labs, Inc. (Scottsdale, PA). The rats were maintained 2 per cage in stainless steel wire mesh cages in a temperature (21-24 C) and humidity (40-60%) controlled room. Feed (Purina chick Startena and Purina rat chow) and water were freely available to the animals at all times. The chickens were randomly placed into 1 control and 2 treatment groups. Each group consisted of 6 subgroups containing 3 birds each. The rats were randomly assigned into one of 2 groups, control or treatment. Each rat group contained 6 subgroups consisting of 2 rats in each.

C. Treatment:

Extracts from the 1988 S. drummondii crop were prepared as described in detail in Experiment 1. The animals were weighed and then gavaged with the S. drummondii extract, if assigned to a treatment group, or with the bicarbonate-azide buffer (BBN), if assigned as a control, for three consecutive days. The volume of extract administered was determined by multiplying the animal's body weight, the dose as percentage of body weight, and the concentration of extract. The treated rats received daily doses equal to 0.5% of body weight and the treated chickens received 0.5% or 0.75% of body weight.

Control animals received equivalent volumes of BBN. The treatment dose (0.5% of body weight) was slightly higher for rats and slightly lower for chickens than used in earlier studies but was selected so that the two animals would receive the same relative dose. Typical clinical signs and necropsy changes were observed with the 0.5% dose in rats but not in chickens. These treated chickens did not appear as clinically-affected as had been observed at higher doses but typical changes at necropsy were still apparent. To insure that maximal S. drummondii induced effects would be studied, a higher dose (0.75% of body weight) was chosen as a second treatment group in chickens.

D. Preparation of hepatic microsomes:

Microsomes were prepared from pooled livers of the subgroups, treated or control, as described in detail in Experiment 1.

E. Protein determination:

The protein concentration of the microsomes was determined as described in detail in Experiment 1.

F. Cytochrome P-450:

The cytochrome P-450 content of the microsomes was measured as described in detail in Experiment 1.

G. Glucose-6-phosphatase:

Glucose-6-phosphatase was measured by determining the rate of release of inorganic phosphate from glucose-6-phosphate as described by Fiske and Subbarow (1925) and modified by Aronson and Touster (1974). The glucose-6-phosphatase reagent assay mixture was prepared by combining solutions of sodium-glucose-6-phosphate (0.1 M, pH 6.5), L-histidine (35mM, pH 6.5), sodium-EDTA (10 mM, pH 7.0) and water in a volume ratio of 2:5:1:1, respectively. The reagent assay mixture was stored frozen to inhibit bacterial growth and thawed to room temperature before use. Microsome suspensions were diluted in 0.125 M KCL to a concentration of approximately 0.20 mg per mL in the dilutions. Glass test tubes were prepared on ice for control, standard, or sample solutions. Three control tubes were utilized in this assay; they contained water, microsomal protein only, or 0.45 mL of the reagent assay mixture. A standard solution contained 0.5 μ mole of sodium phosphate. The standard was demonstrated to be linear in this assay from 0.50 to 2.0 μ mole of sodium phosphate (Appendix 8). Triply distilled water was added to the blank and standard tubes to equal a final volume of 0.5 mL in each tube. The reagent assay mixture (0.45 mL) was transferred to the sample test tubes. All test tubes were preincubated for 5 min in a 37 C shaking water bath. After preincubation, the reactions were started by adding 0.05 mL of diluted microsomes to the sample test tubes and the microsome blank. The reactants, controls,

and standards were then incubated for 20 min in a shaking water bath. The reactions were stopped by plunging the test tubes into ice and adding 2.5 mL of 8% TCA. The acidified samples were centrifuged for 5 min at 3000 g and 5 C to precipitate the protein. Aliquots of the supernatant solutions (2.0 mL) were removed from each test tube and added to clean 18 x 150 millimeter glass test tubes containing 2.3 mL of water. A solution of ammonium molybdate (0.5 mL of 2.5% w/v in 5 N sulfuric acid) was added to the diluted aliquots. The solutions were mixed, and 0.2 mL of Fiske and Subbarow reducer reagent was added. The solutions were again mixed, and after exactly 10 min the optical density of the solutions was read at 660 nm on a spectrophotometer. The glucose-6-phosphatase activity was expressed as the rate of release of inorganic phosphate in μ moles per min per mg of microsomal protein or per nmole of cytochrome P-450. The glucose-6-phosphatase assay was found to be linear with time (15-30 min) and with protein concentration (0.10-0.20 mg/mL) in control microsomes from both chickens and rats (Appendix 9-10).

H. Statistical Analysis:

The significance of difference between control and treated groups was determined using an unpaired Student's t -test. The acceptable level of significance for this study was established at $p < 0.05$.

RESULTS:

The glucose-6-phosphatase activities in hepatic microsomes of chickens and rats treated with Sesbania drummondii are given in Table 6. No differences in the glucose-6-phosphatase activity were observed with Sesbania drummondii treatment at 0.5% of body weight in either the chicken or rat. Sesbania drummondii administered at a higher dosage to chickens (0.75% body weight) also did not significantly alter the glucose-6-phosphate activity. One interesting observation in this study was that control chicken hepatic microsomes had higher glucose-6-phosphate activity than control rat hepatic microsomes.

Table 6: Glucose-6-phosphatase activity of Sesbania drummondii-treated chickens and rats

Treatment group	μ moles Phosphate/min/mg protein
Chickens	
Control (0.5% BW)	0.44 \pm 0.03
<u>Sesbania</u> (0.5% BW)	0.37 \pm 0.04
<u>Sesbania</u> (0.75% BW)	0.47 \pm 0.08
Rats	
Control (0.5% BW)	0.31 \pm 0.04
<u>Sesbania</u> (0.5% BW)	0.29 \pm 0.04

Data are expressed as group means \pm SEM, n = 6. Values were not significantly different ($P < 0.01$) from control values.

DISCUSSION:

Hepatic microsomal glucose-6-phosphatase is located mainly in the endoplasmic reticulum where it catalyzes the terminal stages of both gluconeogenesis and glycogenolysis (Gierow and Jergil, 1982; Burchell et al., 1988). The reaction catalyzed by this enzyme is illustrated by the following equation (Gierow and Jergil, 1982):



Because of its location, glucose-6-phosphatase is commonly used as a marker enzyme for the endoplasmic reticulum. Since the microsomal cytochrome P-450 monooxygenase system is also located in the endoplasmic reticulum, measures of glucose-6-phosphatase activity may be useful in determining the condition of other endoplasmic reticulum-associated enzymes such as the monooxygenase system. Our earlier studies in the rat indicated that S. drummondii-treatment results in a depression of the cytochrome P-450 monooxygenase system. This was shown by decreases in the cytochrome P-450 content and in several cytochrome P-450 mediated enzyme activities. The fact that the glucose-6-phosphatase activity was not altered suggests that the decrease in mixed function monooxygenase activity following S. drummondii-treatment probably was not related to loss of integrity of the microsomal membrane, e.g., via lipid peroxidation.

The significance of higher glucose-6-phosphatase activity in control chicken hepatic microsomes than control rat hepatic microsomes is not known. Perhaps this might relate to the fact that chickens maintain higher blood glucose levels than mammals and therefore require higher levels of this important gluconeogenic enzyme.

B. PHASE II - CHARACTERIZATION OF SESBANIA DRUMMONDII EFFECTS ON THE CATALYTIC PROPERTIES OF THE HEPATIC CYTOCHROME P-450 MONOOXYGENASE SYSTEMS. COMPARISON WITH EFFECTS CAUSED BY PHENOBARBITAL AND β -NAPHTHOFLAVONE.

OBJECTIVES:

1. To compare the effects of Sesbania drummondii, phenobarbital, and β -naphthoflavone on chicken and rat hepatic microsomal metabolism of aminopyrine.
2. To compare the effects of Sesbania drummondii, phenobarbital, and β -naphthoflavone on chicken and rat hepatic microsomal metabolism of aldrin.
3. To compare the effects of Sesbania drummondii, phenobarbital, and β -naphthoflavone on chicken and rat hepatic microsomal metabolism of the alkoxyphenoxazones: methoxyphenoxazone, ethoxyphenoxazone, pentoxyphenoxazone, and benzyloxyphenoxazone.

MATERIALS AND METHODS:

A. Chemicals:

4-Aminophenol (99% pure) and β -naphthoflavone (5,6-benzoflavone, 90-95% pure) were obtained from Sigma Chemical Co. (St. Louis, MO). Aldrin (99% pure) and dieldrin (98% pure) were obtained from Alltech Associates, Inc. (Deerfield, IL). t-Chlordane (99% pure) was obtained from the EPA

(Research Triangle Park, NC). Methoxyphenoxazone, ethoxyphenoxazone, pentoxyphenoxazone, and benzyloxyphenoxazone (all 99% pure) were purchased from Molecular Probes, Inc. (Junction City, OR). Resorufin (75% dye content) was obtained from Aldrich Chemical Co. (Milwaukee, WI). Phenobarbital, sodium injection (65 mg/mL) was obtained from Elkins-Sinn, Inc. (Cherry Hill, NJ). All other chemicals and biochemicals were of research grade or better and were obtained from various commercial sources.

B. Animals:

One day-old White Leghorn chickens were obtained from the Department of Veterinary Science, Louisiana State University, Baton Rouge, LA. At 4 weeks of age, the male birds were separated from the females and used in experimental studies. Ninety of the male chickens (200-400 g) were group housed, 18 per cage, in commercial brooders. The chickens were randomly placed into one of 5 groups, 1 control and 4 treatments. Each group of 18 birds consisted of 6 subgroups containing 3 chickens each.

Forty-eight male Sprague-Dawley rats (300-500 g) were purchased from Hilltop Labs, Inc. (Scottsdale, PA). The rats were maintained 2 per cage in stainless steel wire mesh cages in a temperature (21-24 C) and humidity (40-60%) controlled room. Feed (Purina chick Startena and Purina rat chow) and

water were available ad libitum. The rats were randomly assigned into one of 4 groups, 1 control and 3 treatments. Each rat group of 12 contained 6 subgroups consisting of 2 rats each.

C. Treatment:

Extracts from the 1988 S. drummondii crop were prepared as described in detail in Experiment 1, Phase I. A suspension of β -naphthoflavone (50 mg/mL) was prepared by adding 20 mL of corn oil to 1000 mg of β -naphthoflavone. For 3 days, animals were weighed and then treated with either bicarbonate-azide buffer (BBN), S. drummondii extract, phenobarbital, or β -naphthoflavone. BBN and S. drummondii extract were administered by gavage. The volume of extract given was determined by multiplying the animal's body weight times the dose as percentage of body weight divided by concentration of the extract. Similar volumes of BBN alone were given to the control animals. The S. drummondii-treated rats received daily doses equal to 0.5% of body weight and the chickens received 0.5% or 0.75% of body weight. The treatment dose of 0.5% of body weight was slightly higher for rats and slightly lower for chickens than was used in some of the earlier studies, but was selected so that the two animals would receive the same relative dose. Typical clinical signs and necropsy changes were observed with the 0.5% dose in rats but not chickens. The chickens receiving the 0.5% dose of extract

did not appear as clinically ill as had been observed before, even though typical changes were observed on postmortem examination. In order to insure that maximal S. drummondii-induced effects would be studied, a higher dose (0.75% of body weight) was chosen as an additional treatment group in the chicken.

Phenobarbital was injected through the abdominal musculature of the lower left abdominal quadrant into the peritoneal cavity using a sterile 22 gauge needle. The volume of phenobarbital given was equal to a dosage rate of 60 mg of phenobarbital per kg of body weight. β -Naphthoflavone was also given by injection into the peritoneal cavity but at a dosage rate of 50 mg of β -naphthoflavone per kg of body weight and through an eighteen gauge needle.

D. Preparation of hepatic microsomes:

Microsomes were prepared from pooled livers of the subgroups, treated or control, as described in detail in Experiment 1, Phase I.

E. Protein determination:

The concentration of protein of the microsomes was determined as described in detail in Experiment 1, Phase I.

F. Cytochrome P-450:

The cytochrome P-450 content of the microsomes was measured as described in detail in Experiment 1, Phase I.

G. Hepatic microsomal monooxygenase activities:

1. Aminopyrine N-demethylase:

The microsomal-mediated metabolism of aminopyrine was measured by analysis of the formation of formaldehyde as shown in Figure 3. Optimal assay conditions with respect to incubation time, concentration of microsomal protein, and substrate concentration were empirically determined for control chicken and rat hepatic microsomes (Appendices 11-12). These conditions (Table 7) were utilized in determining the aminopyrine N-demethylase activities of the control, S. drummondii-, phenobarbital-, and the β -naphthoflavone-treated animals. The components of the reaction mixture were the same as described in detail in Experiment 1, Phase I. However in these assays, the reactions were stopped by the addition of 1 mL of 20% rather than 70% trichloroacetic acid.

2. Aldrin epoxidase:

The microsomal-mediated metabolism of aldrin was determined by analysis of the formation of dieldrin (Figure 4). Optimal assay conditions with respect to incubation time, microsomal protein concentration, and substrate concentration were empirically determined for control chicken and rat hepatic

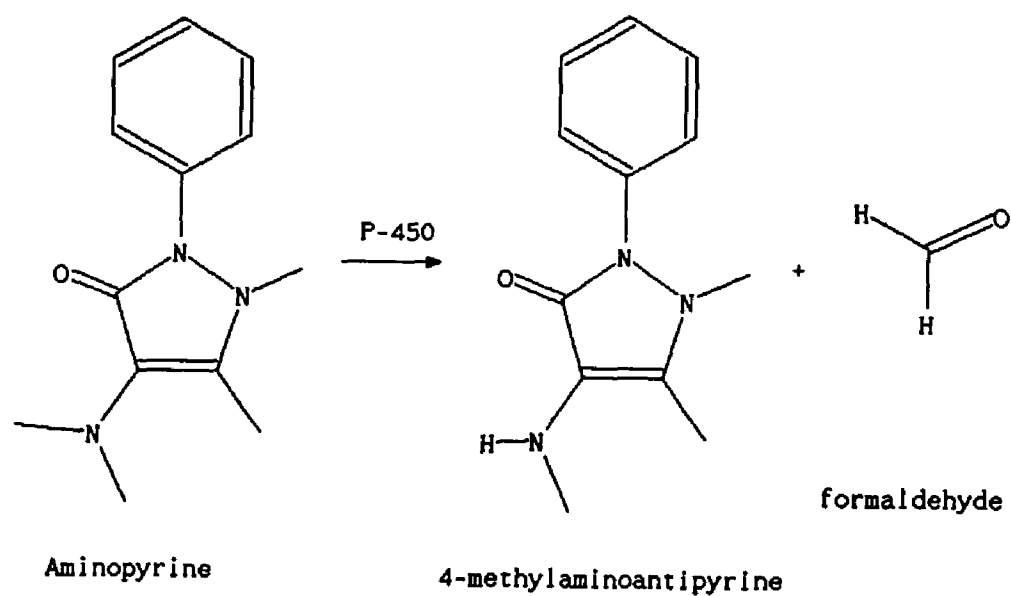


Figure 3. The cytochrome P-450 monooxygenase-catalyzed N-demethylation of aminopyrine.

Table 7: Optimized conditions for the assay of aminopyrine N-demethylase activity in hepatic microsomes from control chickens and rats

Condition	Chicken	Rat
Time (min)	15	15
Protein concentration (mg/mL)	0.5	0.5
Substrate concentration (mM)	10.0	10.0

All assays were performed at 37°C. The assays were conducted in duplicate using a single control chicken or rat hepatic microsomal preparation over a range of 5 - 6 protein concentrations, 5 incubation times, and 6 aminopyrine substrate concentrations. The protein concentration and incubation times yielded maximal product formation in the linear range (see Appendices 11-12).

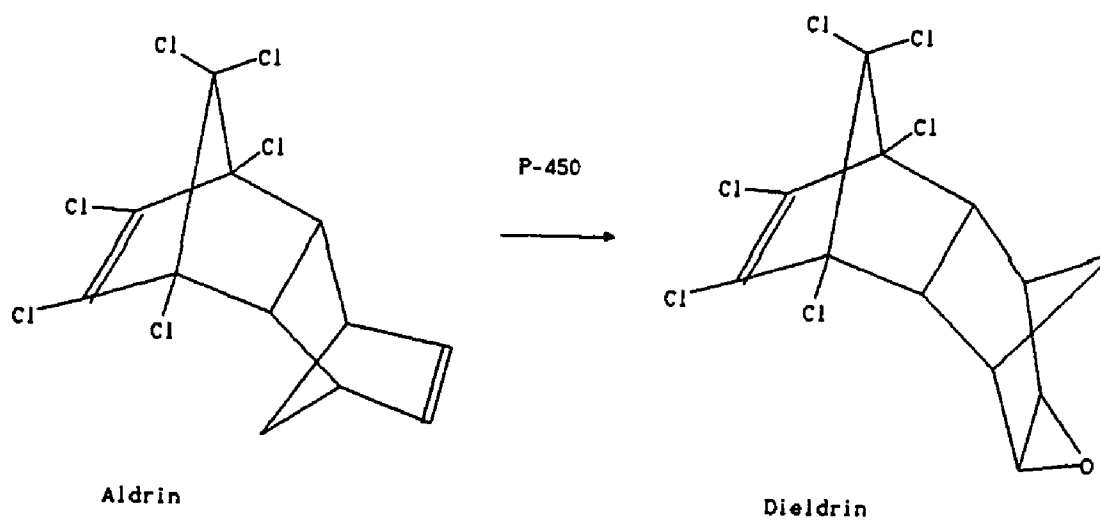


Figure 4. Cytochrome P-450 monooxygenase-mediated epoxidation of aldrin.

Table 8: Optimized conditions for the assay of aldrin epoxidase activity in hepatic microsomes from control chickens and rats

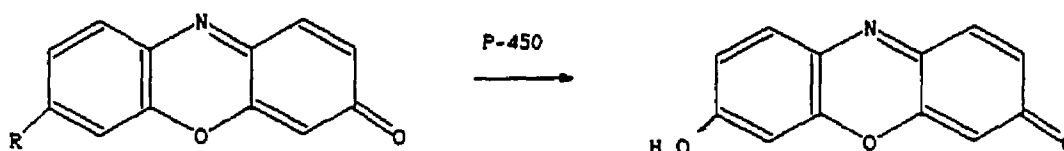
Condition	Chicken	Rat
Time (min)	15	15
Protein concentration (mg/mL)	0.2	0.2
Substrate concentration (μ M)	50.0	50.0

All assays were performed at 37°C. The assays were conducted in duplicate using a single control chicken or rat hepatic microsomal preparation over a range of 4 - 5 protein concentrations, 5 - 6 incubation times, and 6 aldrin substrate concentrations. The protein concentrations and incubation times yielded maximal product formation in the linear range (see Appendices 13-14).

microsomes (Appendices 13-14). These conditions (Table 8) were utilized in determining the aldrin epoxidase activities of control, *S. drummondii*-, phenobarbital-, and β -naphthoflavone-treated animals. The components of the reaction mixture and the quantitation of dieldrin were as described in detail in Experiment 1, Phase I. However, two modifications to the extraction procedure were made in the assay. First, the extraction solution was changed from hexane to 2,2,4-trimethylpentane to reduce the possibility of solvent evaporation. The second modification was the addition of an internal standard, t-chlordane (0.2 ppm), to the 2,2,4-trimethylpentane extraction solution to minimize instrument error due to the injection of unequal sample aliquots.

3. Alkoxyphenoxazone O-dealkylase:

The O-dealkylations of methoxy-, ethoxy-, pentoxy-, and benzyloxyphenoxazone were measured by the fluorometric methods of Burke and Mayer (1983) and that of Burke *et al.* (1985). These reactions are described in Figure 5. Microsome dilutions were prepared in 0.125 M KCL. Stock solutions (1mM) of the alkoxyphenoxazone substrates were placed into dimethylsulfoxide (water was removed over 3A molecular sieves) and sonicated in glass reagent vials. A stream of nitrogen gas was passed over the solutions, the vials were capped, covered with aluminum foil, and stored frozen (0 C). Stock solutions were thawed to room temperature on the day of an



R = H	phenoxazine	
R = OH	hydroxyphenoxazine	(Resorufin)
R = CH ₃ O	methoxyphenoxazine	(MPOD)
R = C ₂ H ₅ O	ethoxyphenoxazine	(EPOD)
R = C ₅ H ₁₁ O	pentoxyphenoxazine	(PPOD)
R = C ₆ H ₅ CH ₂ O	benzyloxyphenoxazine	(BPOD)

Figure 5. Cytochrome P-450 monooxygenase-mediated O-dealkylation of the alkoxyphenoxazines.

assay and then refrozen. Optimal assay conditions (for each of the alkoxyphenoxazone O-dealkylations) with respect to incubation time and concentration of microsomal protein were determined in control, *S. drummondii*-, phenobarbital-, and β -naphthoflavone-treated chicken and rat hepatic microsomes in order to insure that the reactions were conducted under conditions of protein and time linearity (Appendices 15-22). These conditions are summarized in Tables 9-12. The reaction mixtures contained microsomal protein, 5 μ M substrate (10 μ L of 1mM stock solution), 0.1 M potassium phosphate buffer (pH 7.6), and 250 μ M NADPH in a total volume of 2 mL. A cuvette containing the mixture was placed into the sample compartment of an Aminco-Bowman spectrophotofluorometer (Travenol Laboratories, Inc., Deerfield, IL). The compartment was kept warm (37 C) with a circulating temperature bath. The cuvettes were preincubated for 2 min, the reactions started with NADPH, and the mixtures incubated for another 2 min to assure that maximal linear velocities were obtained. The reaction was then followed for an additional 1 to 4 min depending on the rate of resorufin production. Reaction rates were measured directly from the increasing fluorescence of the reaction mixture at 530 nm excitation wavelength and 585 nm emission wavelength. The resorufin concentration was quantified by standard addition with a 2-20 μ L aliquot of resorufin (25 μ M in methanol) added to the cuvette after a suitable reaction period.

Table 9: Optimized conditions for the assay of methoxyphenoxazone O-dealkylase activity in hepatic microsomes from control chickens and rats

Animal	Treatment	Time (min)	Protein conc. (mg/mL)
Chicken	Control	2	0.100
	<u>Sesbania</u>	2	0.050
	Phenobarbital	2	0.100
	β -naphthoflavone	2	0.050
Rat	Control	3	0.100
	<u>Sesbania</u>	4	0.100
	Phenobarbital	2	0.050
	β -naphthoflavone	2	0.050

All assays were performed at 37°C. The assays were conducted using a single control or treated chicken or rat hepatic microsomal preparation over a range of 2 protein concentrations and 5 incubation times. The protein concentrations and incubation times yielded maximal product formation in the linear range (see Appendix 15-16).

Table 10: Optimized conditions for the assay of ethoxyphenoxazone O-dealkylase activity in hepatic microsomes from control chickens and rats

Animal	Treatment	Time (min)	Protein conc. (mg/mL)
Chicken	Control	2	0.0250
	<u>Sesbania</u>	2	0.0250
	Phenobarbital	2	0.0250
	β -Naphthoflavone	2	0.0125
Rat	Control	2	0.0125
	<u>Sesbania</u>	2	0.0250
	Phenobarbital	2	0.0125
	β -Naphthoflavone	2	0.0125

All assays were performed at 37°C. The assays were conducted using a single control or treated chicken or rat hepatic microsomal preparation over a range of 2 protein concentrations and 5 incubation times. The protein concentrations and incubation times yielded maximal product formation in the linear range (see Appendices 17-18).

Table 11: Optimized conditions for the assay of pentoxyphenoxazone O-dealkylase activity in hepatic microsomes from control chickens and rats

Animal	Treatment	Time (min)	Protein conc. (mg/mL)
Chicken	Control	3	0.100
	<u>Sesbania</u>	3	0.100
	Phenobarbital	3	0.200
	β -Naphthoflavone	2	0.100
Rat	Control	3	0.100
	<u>Sesbania</u>	4	0.100
	Phenobarbital	1	0.025
	β -Naphthoflavone	2	0.050

All assays were performed at 37°C. The assays were conducted using a single control or treated chicken or rat hepatic microsomal preparation over a range of 2 protein concentrations and 6 - 8 incubation times. The protein concentrations and incubation times yielded maximal product formation in the linear range (see Appendices 19-20).

Table 12: Optimized conditions for the assay of benzyloxyphenoxazone O-dealkylase activity in hepatic microsomes from control chickens and rats

Animal	Treatment	Time (min)	Protein conc (mg/mL)
Chicken	Control	2	0.1000
	<u>Sesbania</u>	2	0.1000
	Phenobarbital	2	0.0500
	B-Naphthoflavone	2	0.0250
Rat	Control	2	0.0500
	<u>Sesbania</u>	2	0.1000
	Phenobarbital	1	0.0125
	B-Naphthoflavone	2	0.0500

All assays were performed at 37°C. The assays were conducted using a single control or treated chicken or rat hepatic microsomal preparation over a range of 2 protein concentrations and 4 - 6 incubation times. The protein concentrations and incubation times yielded maximal product formation in the linear range (see Appendices 21-22).

H. Statistical Analyses:

Statistical analyses of the data were performed using an analysis of variance (ANOVA) among the chicken or rat experimental groups. This was accomplished by determining the mean and standard deviation for each group. Differences among the means and standard deviations were tested for significance using Scheffe's multiple range test (Snedecor and Cochran, 1980). These calculations were carried out on an IBM-PC/AT computer (IBM, Dallas, TX) using the STATGRAPHICS^R program (version 2.1) by Statistical Graphics Corp. (Rockville, MD). The acceptable level of significance for this study was established at $p < 0.05$.

RESULTS:

Thorough characterization of xenobiotic-induced changes on the catalytic function of the cytochrome P-450 monooxygenase system requires analysis of both the specific activity (in terms of mg microsomal protein) and the molar activity or turnover number (in terms of nmoles of cytochrome P-450). Therefore, before these activities could be determined, it was necessary first to determine the protein concentration and the cytochrome P-450 content of the control and treated chicken and rat hepatic microsomes.

The effect of treatment with Sesbania drummondii, phenobarbital, and β -naphthoflavone on the cytochrome P-450 content of chicken and rat hepatic microsomes is shown in Table 13. The Sesbania drummondii-induced effects on cytochrome P-450 content confirm the changes seen in the preliminary study (Table 2). Sesbania drummondii treatment induced a significant 2-fold increase in chicken and a 2-fold decrease in rat cytochrome P-450 content. The magnitude of induction of chicken cytochrome P-450 did not appear to be improved by increasing the dosage. Chickens treated with the higher dosage (0.75% of body weight) had an induction of cytochrome P-450 content nearly identical to that of the lower treatment dosage (0.5% of body weight).

Table 13: The effect of Sesbania drummondii, phenobarbital, and β -naphthoflavone on chicken and rat hepatic cytochrome P-450 specific content and absorbance maxima.

Treatment	Cytochrome P-450	
	Concentration (nmoles/mg protein)	λ max (nm)
Chicken		
Control	0.17 ± 0.02^a	450.9 ± 0.7^a
<u>Sesbania</u> (0.5% BW)	0.33 ± 0.04^b	450.6 ± 0.6^a
<u>Sesbania</u> (0.75% BW)	0.32 ± 0.04^b	450.6 ± 0.4^a
Phenobarbital	0.36 ± 0.03^b	450.6 ± 0.3^a
β -Naphthoflavone	0.63 ± 0.06^c	448.4 ± 0.4^b
Rat		
Control	0.86 ± 0.11^a	449.5 ± 0.2^{ab}
<u>Sesbania</u> (0.5% BW)	0.48 ± 0.04^b	449.6 ± 0.6^a
Phenobarbital	2.14 ± 0.24^c	449.1 ± 0.3^b
β -Naphthoflavone	1.22 ± 0.06^d	447.4 ± 0.3^c

Values are the mean \pm SEM, n = 6.

Means with like superscripts within the same species are not significantly different (P > 0.05).

Phenobarbital and β -naphthoflavone treatments resulted in significant increases of cytochrome P-450 content in both chicken and rat hepatic microsomes. The magnitude of the increase in cytochrome P-450 content indicated species similarities in response to phenobarbital but species differences in response to β -naphthoflavone. Approximately 2 to 2.5-fold increases in cytochrome P-450 content were found in both chickens and rats treated with phenobarbital. However, treatment with β -naphthoflavone resulted in a nearly 4-fold increase in chicken but only a 1.5-fold increase in rat cytochrome P-450 content. The magnitude of the induction of cytochrome P-450 content with Sesbania drummondii treatment of chickens closely paralleled that of phenobarbital. Neither of the Sesbania drummondii chicken treatment groups significantly differed from the phenobarbital treatment group.

In addition to changes in enzyme activity, the cytochrome P-450 inducers can also change the enzyme's spectral properties. The wavelength at which the carbon monoxide complex of reduced cytochrome P-450 produces peak absorption is a useful indicator of qualitative changes in cytochrome P-450 content. The effects of Sesbania drummondii, phenobarbital, and β -naphthoflavone on this parameter (λ max) are also listed in Table 13. Treatment with β -naphthoflavone in chickens and rats produced the characteristic blue shift in the peak absorption by 2 nm, i.e., to 448nm. Sesbania drummondii-

treatment of chickens and rats did not appear to alter the wavelength maximum.

Aminopyrine N-demethylase:

The aminopyrine N-demethylase activities of chicken and rat hepatic microsomes from animals treated with Sesbania drummondii, phenobarbital, and β -naphthoflavone are displayed in Table 14. The aminopyrine N-demethylase specific activities in chickens and rats treated with Sesbania drummondii verify the results of the preliminary study (Table 3). A 2-fold increase in chicken and a 2-fold decrease in rat specific activity were again observed with Sesbania drummondii. Chickens administered Sesbania drummondii at the greater percentage of body weight (0.75%) had significantly higher specific activities than chickens treated at 0.5% of body weight. Species differences were observed in the aminopyrine N-demethylase specific activities of hepatic microsomes from animals treated with the classic inducers, phenobarbital and β -naphthoflavone. Treatment with phenobarbital resulted in increased activities in both species. However, treatment with β -naphthoflavone increased the activity only in the chicken. The magnitude of the increase in specific activity was similar for both phenobarbital, β -naphthoflavone and Sesbania drummondii treatments of chickens.

Table 14: The effect of Sesbania drummondii, phenobarbital, and β -naphthoflavone on aminopyrine N-demethylase activity in chicken and rat hepatic microsomes.

Treatment	nmoles Formaldehyde formed/ min	
	per mg protein	per nmole P-450
Chicken		
Control	3.14 ± 0.30^a	19.03 ± 2.58^a
<u>Sesbania</u> (0.5% BW)	6.15 ± 1.10^b	18.43 ± 1.12^a
<u>Sesbania</u> (0.75% BW)	8.57 ± 1.89^c	27.11 ± 3.82^b
Phenobarbital	8.13 ± 0.92^{bc}	22.71 ± 3.16^{ab}
β -Naphthoflavone	6.48 ± 1.01^{bc}	10.33 ± 1.46^c
Rat		
Control	3.76 ± 0.58^a	4.35 ± 0.64^a
<u>Sesbania</u> (0.5% BW)	1.71 ± 0.32^b	3.61 ± 0.70^a
Phenobarbital	11.32 ± 1.25^c	5.31 ± 0.37^b
β -Naphthoflavone	3.14 ± 0.38^a	2.58 ± 0.25^c

Values are the mean \pm SEM, n = 6.

Means with like superscripts within the same species are not significantly different ($P > 0.05$).

Similarities among some groups and differences among others were observed in the treatment-induced relative changes in molar and specific activity of aminopyrine N-demethylase. The molar activity, unlike the specific activity, of chickens and rats treated with Sesbania drummondii at 0.5% of body weight was not significantly different from controls. Treatment of chickens with Sesbania drummondii at 0.75% of body weight, however, resulted in a molar activity significantly different from control and the low dosage Sesbania drummondii treatment. The molar activity of the high dosage Sesbania drummondii treatment overlapped the molar activity of phenobarbital, a finding also observed with specific activity. The aminopyrine N-demethylase molar activity of phenobarbital-treated chickens differed from controls in rat but not chicken hepatic microsomes. β -Naphthoflavone produced significantly different aminopyrine N-demethylase molar activities in both chickens and rats.

Aldrin epoxidase:

The aldrin epoxidase activities in chicken and rat hepatic microsomes from animals treated with Sesbania drummondii, phenobarbital, and β -naphthoflavone are shown in Table 15. The aldrin epoxidase specific activities of chickens and rats treated with Sesbania drummondii at 0.5% of body weight are similar to the findings from the preliminary study (Table 3). Sesbania drummondii-treatment induced an increase in chicken

Table 15: The effect of *Sesbania drummondii*, phenobarbital, and β -naphthoflavone on aldrin epoxidase activity in chicken and rat hepatic microsomes.

Treatment	nmoles Dieldrin produced / min	
	per mg protein	per nmole P-450
Chicken		
Control	1.70 \pm 0.18 ^a	10.29 \pm 1.27 ^a
<i>Sesbania</i> (0.5% BW)	5.20 \pm 1.29 ^b	15.64 \pm 2.36 ^b
<i>Sesbania</i> (0.75% BW)	6.81 \pm 1.30 ^{bc}	21.58 \pm 2.81 ^c
Phenobarbital	8.43 \pm 1.80 ^c	23.57 \pm 5.89 ^c
β -Naphthoflavone	1.65 \pm 0.28 ^a	2.63 \pm 0.38 ^d
Rat		
Control	4.58 \pm 0.30 ^a	5.40 \pm 0.55 ^a
<i>Sesbania</i> (0.5% BW)	2.01 \pm 0.18 ^b	4.24 \pm 0.17 ^b
Phenobarbital	23.54 \pm 2.24 ^c	11.03 \pm 0.28 ^c
β -Naphthoflavone	2.07 \pm 0.38 ^b	1.70 \pm 0.32 ^d

Values are the mean \pm SEM, n = 6.

Means with like superscripts within the same species are not significantly different (P > 0.05).

and a decrease in rat aldrin epoxidase specific activities. The magnitude of the decrease in the rat was 2-fold as had been observed in the preliminary study. However, the magnitude of the increase in chicken specific activity was 3-fold, less than the 4-fold increase observed previously. The chickens of the preliminary study had been treated with Sesbania drummondii extract at a dosage of 0.80% of their body weight. Increasing the dosage of Sesbania drummondii to 0.75% in the chicken increased induction to 4-fold.

The greatest increase of aldrin epoxidase specific activity in all the treatment groups from both species was observed with phenobarbital. Approximately 5-fold inductions were observed in both chickens and rats treated with phenobarbital. Species differences in aldrin epoxidase specific activity were observed with β -naphthoflavone treatment. No change in activity was observed in chickens, whereas rats exhibited a 2-fold decrease in activity in the β -naphthoflavone treatment groups. The aldrin epoxidase specific activities of Sesbania drummondii- and β -naphthoflavone-treated rats were not significantly different.

When aldrin epoxidase activity was expressed in terms of nmole cytochrome P-450, significant differences were observed among controls, Sesbania drummondii, phenobarbital, and β -naphthoflavone treatment groups in both chickens and rats.

Only the two Sesbania drummondii-treatment groups in chickens were not significantly different.

Alkoxyphenoxazone O-dealkylases:

Species differences were observed for both specific activities and molar activities of the four alkoxyphenoxazone O-dealkylases. These differences were observed in response to treatment with Sesbania drummondii as well as with the two chemical inducers, phenobarbital and β -naphthoflavone.

Methoxyphenoxazone O-dealkylase:

Methoxyphenoxazone O-dealkylase activity of chicken and rat hepatic microsomes from animals treated with Sesbania drummondii, phenobarbital and β -naphthoflavone are shown in Table 16. The methoxyphenoxazone O-dealkylase specific activities of both chickens and rats treated with Sesbania drummondii were different from controls. A 2-fold increase in chickens' and a 3-fold decrease in rats' specific activity were observed in animals treated with Sesbania drummondii at 0.5% of body weight. A slight, but not significant, increase in specific activity occurred in chickens treated with Sesbania drummondii at 0.75% of body weight over those treated at the lower dosage. Phenobarbital treatment resulted in a slight, but not significant, increase in specific activity in treated chickens and a 3-fold increase in treated rats. The greatest induction of methoxyphenoxazone O-dealkylase

Table 16: Methoxyphenoxazone O-dealkylase activity of *Sesbania drummondii*, phenobarbital and 8-naphthoflavone-treated chickens and rats

Treatment	pmoles resorufin produced / min	
	per mg protein	per nmole P-450
Chicken		
Control	88 ± 16 ^a	526 ± 81 ^{ab}
<i>Sesbania</i> (0.5% BW)	185 ± 47 ^{bc}	571 ± 165 ^{ab}
<i>Sesbania</i> (0.75% BW)	223 ± 57 ^c	707 ± 152 ^b
Phenobarbital	142 ± 28 ^{ab}	394 ± 71 ^a
8-Naphthoflavone	1295 ± 206 ^d	2064 ± 279 ^c
Rat		
Control	55 ± 6 ^a	64 ± 7 ^a
<i>Sesbania</i> (0.5% BW)	16 ± 4 ^b	35 ± 9 ^b
Phenobarbital	177 ± 32 ^c	83 ± 11 ^c
8-Naphthoflavone	522 ± 120 ^d	430 ± 103 ^d

Values are the mean ± SEM, n = 6.

Means with like superscripts within the same species are not significantly different (P > 0.05).

activity, however, resulted from β -naphthoflavone treatment. Increases of 15-fold in chicken and 9-fold in rat specific activities occurred with β -naphthoflavone treatment.

Fewer differences between treatment groups were evident in methoxyphenoxazone O-dealkylase molar activities. In the chicken hepatic microsomes, only treatment with β -naphthoflavone resulted in molar activity significantly different from the controls and other treatment groups. In contrast, the methoxyphenoxazone O-dealkylase molar activities in rat hepatic microsomes from each of the treatment groups and controls were significantly different from each other.

Ethoxyphenoxazone O-dealkylase:

The ethoxyphenoxazone O-dealkylase activity of hepatic microsomes from chickens and rats treated with Sesbania drummondii, phenobarbital, and β -naphthoflavone are listed in Table 17. Fewer changes in both specific and molar activities were induced in the treated chickens for ethoxyphenoxazone O-dealkylation than in the 3 other alkoxyphenoxazone O-dealkylases. The effect of Sesbania drummondii treatment on ethoxyphenoxazone O-dealkylase activity was different from the observations of the preliminary study (Table 3). The ethoxyphenoxazone O-dealkylase specific activities of chicken hepatic microsomes were not significantly different between controls and birds treated with Sesbania drummondii and

Table 17: Ethoxyphenoxazone O-dealkylase activity of Sesbania drummondii, phenobarbital, and β -naphthoflavone-treated chickens and rats

Treatment	pmoles resorufin produced / min	
	per mg protein	per nmole P-450
Chicken		
Control	388 \pm 91 ^a	2401 \pm 857 ^a
<u>Sesbania</u> (0.5% BW)	479 \pm 106 ^a	1482 \pm 377 ^a
<u>Sesbania</u> (0.75% BW)	463 \pm 108 ^a	1465 \pm 278 ^a
Phenobarbital	594 \pm 163 ^a	1673 \pm 544 ^a
β -Naphthoflavone	10652 \pm 882 ^b	17070 \pm 2000 ^b
Rat		
Control	371 \pm 118 ^a	427 \pm 99 ^a
<u>Sesbania</u> (0.5% BW)	300 \pm 75 ^a	638 \pm 177 ^b
Phenobarbital	1634 \pm 167 ^b	769 \pm 81 ^b
β -Naphthoflavone	6687 \pm 1232 ^c	5508 \pm 1089 ^c

Values are the mean \pm SEM, n = 6.

Means with like superscripts within the same species are not significantly different ($P > 0.05$).

phenobarbital. Only treatment with β -naphthoflavone resulted in significant induction (27-fold) of specific activity in chickens.

A pattern similar to the ethoxyphenoxazone O-dealkylase specific activities of chickens was observed in the rat for treatments with Sesbania drummondii and β -naphthoflavone. Sesbania drummondii treatment of rats failed to significantly reduce the ethoxyphenoxazone O-dealkylase specific activity and β -naphthoflavone treatment markedly increased the specific activity. The magnitude of the β -naphthoflavone-induced increase in the rat however, was less than observed in the chicken (only 18-fold). The rat was also different from the chicken with regard to phenobarbital treatment. The rat, but not the chicken, exhibited a moderate induction of specific activity of 4-fold with phenobarbital treatment.

The ethoxyphenoxazone O-dealkylase molar activities of the treated groups followed a pattern similar to the specific activities in the chicken but not the rat. Only β -naphthoflavone-treated chickens exhibited a different ethoxyphenoxazone O-dealkylase molar activity, whereas controls, Sesbania drummondii-, and phenobarbital-treated chickens yielded molar activities that were not significantly different. In the rat, only β -naphthoflavone treatment exhibited a molar activity concurrent with the change in

specific activity. The ethoxyphenoxazone O-dealkylase molar activity of the β -naphthoflavone-treated rat was markedly different from all other groups. Treatment with Sesbania drummondii produced a molar activity significantly different from controls as was seen with the specific activity. This activity was also statistically similar to treatment with phenobarbital, a finding not observed for specific activity.

Pentoxifyphenoxazone O-dealkylase:

The pentoxifyphenoxazone O-dealkylase activity in hepatic microsomes from chickens and rats treated with Sesbania drummondii, phenobarbital, and β -naphthoflavone is given in Table 18. Pentoxifyphenoxazone O-dealkylase activity was the only cytochrome P-450-mediated enzyme activity in which both chickens and rats treated with Sesbania drummondii expressed the same relative change in activity compared to controls. Sesbania drummondii administered to chickens at 0.5% of body weight caused a slight decrease in specific activity that was not significantly different from controls. The pentoxifyphenoxazone O-dealkylase specific activity of chickens treated at 0.75% of body weight and rats treated at 0.5% of body weight with Sesbania drummondii were both significantly lower than the corresponding control activity. Rats, however, showed a greater decrease in specific activity than the chickens (3-fold versus 1.5-fold, respectively).

Table 18: Pentoxyphenoxazone O-dealkylase activity of Sesbania drummondii, phenobarbital, and β -naphthoflavone-treated chickens and rats.

	pmoles resorufin produced / min			
Treatment	per mg protein		per nmole P-450	
Chicken				
Control	10 ±	2 ^a	60 ±	7 ^a
<u>Sesbania</u> (0.5% BW)	9 ±	1 ^a	28 ±	7 ^b
<u>Sesbania</u> (0.75% BW)	6 ±	2 ^b	20 ±	5 ^{bc}
Phenobarbital	4 ±	1 ^b	13 ±	2 ^c
β-Naphthoflavone	86 ±	9 ^c	137 ±	12 ^d
Rat				
Control	18 ±	4 ^a	21 ±	5 ^a
<u>Sesbania</u> (0.5% BW)	6 ±	1 ^b	12 ±	1 ^b
Phenobarbital	3548 ±	384 ^c	1666 ±	153 ^c
β-Naphthoflavone	82 ±	12 ^d	68 ±	8 ^d

Values are the mean \pm SEM, n = 6.

Means with like superscripts within the same species are not significantly different (P > 0.05).

Phenobarbital treatment of rats resulted in a very large induction of pentoxyphenoxazone O-dealkylase specific activity (197-fold) over that of controls. In contrast, phenobarbital treatment of chickens resulted in an actual decrease in the pentoxyphenoxazone O-dealkylase specific activity. The decreased specific activity with phenobarbital treatment of the chicken was statistically similar to the decrease observed with Sesbania drummondii treatment at 0.75% of body weight. The pentoxyphenoxazone O-dealkylase specific activity of both chickens and rats treated with β -naphthoflavone were significantly higher than controls. β -Naphthoflavone treatment of chickens induced the pentoxyphenoxazone O-dealkylase specific activity nearly 9-fold, higher than any other treatment. The β -naphthoflavone-induced increase in the rat, however, was only 4-fold.

The molar activities of pentoxyphenoxazone O-dealkylase followed a pattern similar to the specific activities in the rat, but not the chicken. In the rat, the pentoxyphenoxazone O-dealkylase molar activity was distinctly different among control, S. drummondii, phenobarbital, and β -naphthoflavone treatments. The chicken pentoxyphenoxazone O-dealkylase molar activities showed statistically distinct differences among control, phenobarbital and β -naphthoflavone-treated groups and similarities among phenobarbital and S. drummondii (0.75%).

Benzyloxyphenoxazone O-dealkylase:

Benzyloxyphenoxazone O-dealkylase activity of chicken and rat hepatic microsomes from animals treated with Sesbania drummondii, phenobarbital, and β -naphthoflavone are shown in Table 19. Treatment of chickens with Sesbania drummondii at both dosages resulted in benzyloxyphenoxazone specific activities that were not significantly different from controls. The Sesbania drummondii treatment of rats, however, had significantly lower (2-fold) specific activities than controls. The benzyloxyphenoxazone O-dealkylase activities of chickens and rat hepatic microsomes treated with phenobarbital followed a pattern of induction similar to pentoxyphenoxazone O-dealkylation. Phenobarbital treatment of rats resulted in a very large increase in benzyloxyphenoxazone O-dealkylase specific activity (98-fold) while the phenobarbital-treated chicken showed a significant decrease in specific activity (1.5-fold). Treatment of both species with β -naphthoflavone resulted in a marked induction of benzyloxyphenoxazone O-dealkylase specific activities. A 32-fold increase in chicken and a 5-fold increase in rat benzyloxyphenoxazone O-dealkylase specific activity were induced with β -naphthoflavone treatment.

Effects on the molar activities of benzyloxyphenoxazone O-dealkylase observed in chickens and rats treated with Sesbania drummondii were opposite to the specific activities. The

Table 19: Benzyloxyphenoxazone O-dealkylase activity of Sesbania drummondii, phenobarbital, and β -naphthoflavone-treated chickens and rats.

Treatment	pmoles resorufin produced / min	
	per mg protein	per nmole P-450
Chicken		
Control	40 \pm 7 ^a	239 \pm 33 ^a
<u>Sesbania</u> (0.5% BW)	42 \pm 7 ^a	127 \pm 22 ^b
<u>Sesbania</u> (0.75% BW)	43 \pm 9 ^b	136 \pm 19 ^b
Phenobarbital	26 \pm 4 ^b	71 \pm 10 ^c
β -Naphthoflavone	1307 \pm 130 ^c	2088 \pm 190 ^d
Rat		
Control	97 \pm 20 ^a	113 \pm 23 ^a
<u>Sesbania</u> (0.5% BW)	44 \pm 10 ^b	93 \pm 19 ^a
Phenobarbital	9527 \pm 2462 ^c	4431 \pm 940 ^b
β -Naphthoflavone	536 \pm 75 ^d	450 \pm 42 ^c

Values are the mean \pm SEM, n = 6.

Means with like superscripts within the same species are not significantly different (P > 0.05).

chicken benzyloxyphenoxazone O-dealkylase molar activities were significantly different from controls whereas the rats' molar activities were not significantly different with Sesbania drummondii treatment. Both phenobarbital and β -naphthoflavone treatments in chickens and rats resulted in benzyloxyphenoxazone O-dealkylase molar activities distinctly different from controls and Sesbania drummondii-treated animals.

A general overview of the catalytic activities induced by Sesbania drummondii, phenobarbital, and β -naphthoflavone are presented in Figures 6 and 7. These figures illustrate the degree of induction (the reaction rate with induced microsomes divided by the rate with the control microsomes) of the three treatment groups for each of the six cytochrome P-450-mediated activities for both chickens and rats. In the S. drummondii-treated chicken (Figure 6), the catalytic properties were similar to the catalytic properties of chickens treated with phenobarbital and were different from the catalytic properties of chickens treated with β -naphthoflavone. Additional support for the similarities between phenobarbital and S. drummondii are added by the similar spectral properties. The total cytochrome P-450 specific contents and the absorbance maxima were similar between these two treatments.

The catalytic properties of Sesbania drummondii-treated rats

presented in Figure 7 are clearly different from those of phenobarbital and β -naphthoflavone treatments. These differences were also evident in the spectral properties of these treatments.

**Induction of CP450-mediated activities
in hepatic microsomes from chickens
treated with S.drum, PB and BNF**

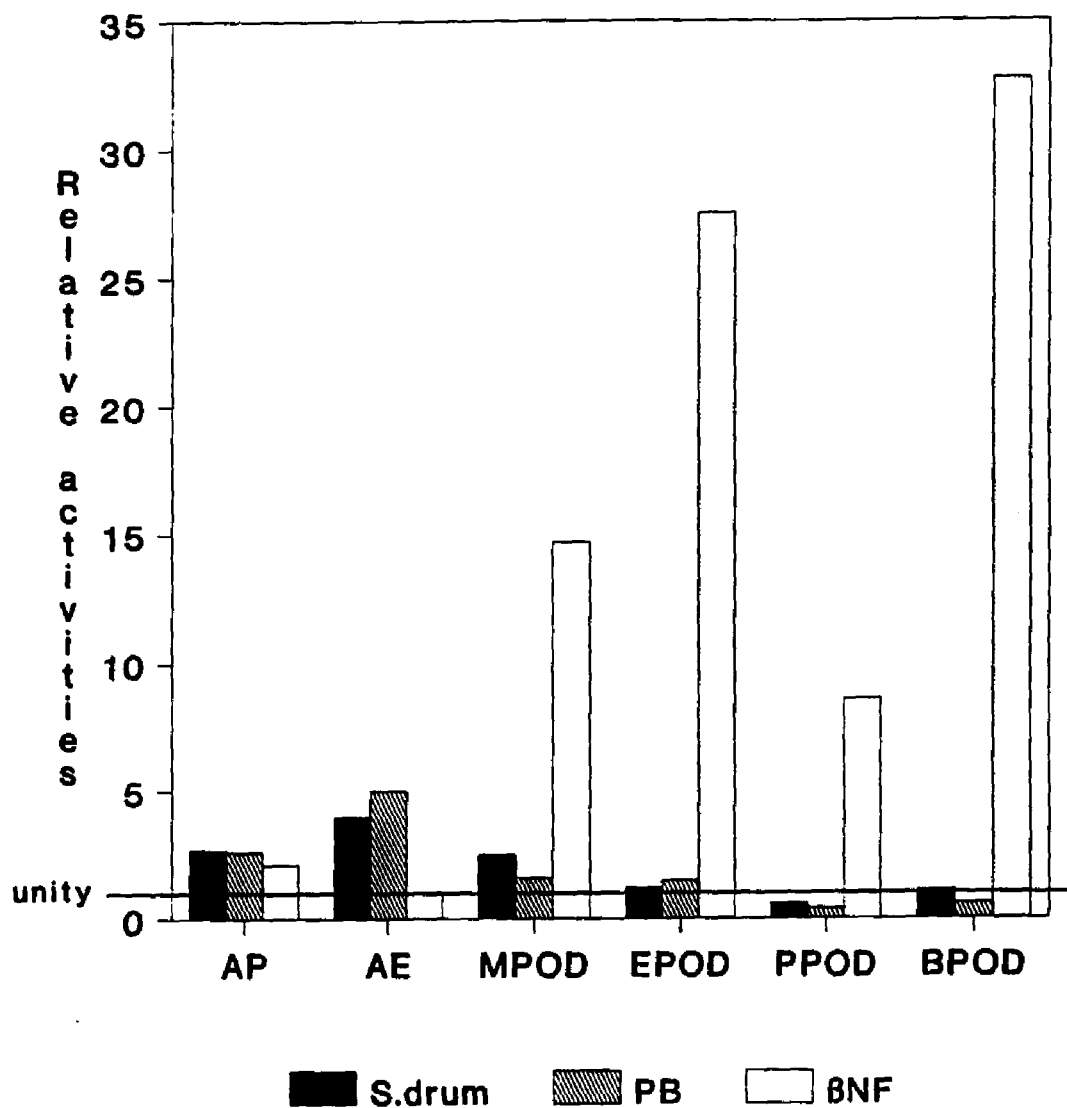


Figure 6. Degree of induction of cytochrome P-450-mediated activities of hepatic microsomes from chickens treated with *Sesbania drummondii*, phenobarbital, and β -naphthoflavone.

**Induction of CP450-mediated activities
in hepatic microsomes from rats
treated with S.drug, PB and 8NF**

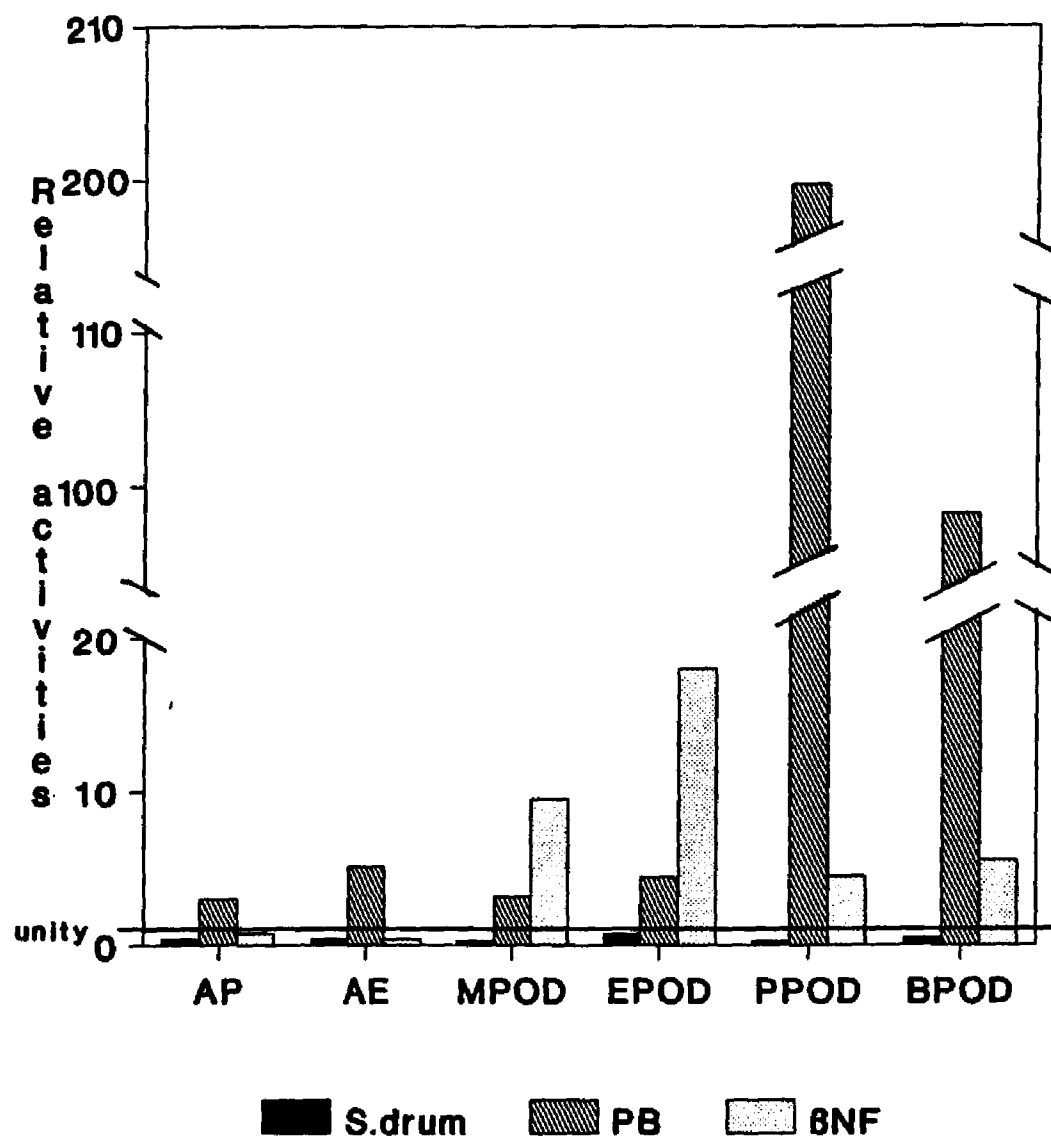


Figure 7. Degree of induction of cytochrome P-450-mediated activities of hepatic microsomes from rats treated with *Sesbania drummondii*, phenobarbital, and 8-naphthoflavone.

DISCUSSION:

The cytochrome P-450 isozymes exhibit a unique overall profile of substrate selectivity. Examining the catalytic properties of the cytochrome P-450-dependent monooxygenase system in hepatic microsomes following treatment is a useful means of characterizing the effects of treatment on the cytochrome P-450 isozyme profile. However, there are limitations to catalytic characterization because many of the cytochrome P-450 isozymes exhibit overlapping substrate selectivities (Ryan and Levin, 1990).

In order to evaluate the effect of a new compound on the hepatic cytochrome P-450 monooxygenase system, it is essential to simultaneously compare the effects with those induced by positive and negative controls in the same animal system. This is because of the many factors related to the host (sex, age, health) and its environment (temperature, humidity, diet) that are known to alter the cytochrome P-450 monooxygenase system (Sipes and Gandolfi, 1986).

In our preliminary investigations, we compared the effects of Sesbania drummondii-treatment on the hepatic cytochrome P-450 monooxygenase systems in chickens and rats with the effects in negative controls only. Therefore, our understanding of the changes induced by S. drummondii was limited because comparisons with known inducers of this system could only be

made with literature reports. This is less of a problem in the rat due to the abundance of published data. However, in the chicken, there are few reports and these are usually limited to studies of chicken embryos or adults.

Our goal in this study was to compare the effects of Sesbania drummondii treatment on the catalytic properties of the hepatic cytochrome P-450 monooxygenase systems of chickens and rats to the effects induced by two classical cytochrome P-450 inducers, phenobarbital and β -naphthoflavone. We studied six cytochrome P-450-mediated activities of varying degrees of selectivity and specificity. In addition, we obtained general information about the effects of the treatments on the cytochrome P-450 monooxygenase system by performing spectral determinations of cytochrome P-450.

Spectral determinations of cytochrome P-450 allow for convenient measures of cytochrome P-450 specific content and absorbance maximum. These measures provide some general information about the cytochrome P-450 system but have limited utility for identifying specific forms of cytochrome P-450.

Most of the inducers of the cytochrome P-450 system increase the specific content of cytochrome P-450. However, this is not true for all cytochrome P-450 inducers. A few inducers increase the amount of some of the cytochrome P-450 forms

while decreasing the content of others resulting in no overall change in the total specific content (Ryan and Levin, 1990). The two classical inducers, phenobarbital and β -naphthoflavone, increased the cytochrome P-450 content in both chickens and rats. Sesbania drummondii treatment also resulted in an increase in chicken but a decrease in rat total cytochrome P-450 specific content. The magnitude of the S. drummondii increase in the cytochrome P-450 specific content was 2-fold, virtually the same as the increase observed in chickens treated with phenobarbital. This finding differed from published reports in which phenobarbital treatment of chickens increased the cytochrome P-450 content by 3-4 fold (Strittmater and Umberger, 1969; Powis et al., 1976; Darby et al., 1985; Lorr and Bloom, 1987). The reason our phenobarbital-treated chickens showed smaller inductions is not known. The age of the chickens should not have been a factor because phenobarbital induction of cytochrome P-450 of greater than 3-fold has been reported in chickens from embryos to adults (Powis et al., 1976; Lapadula et al., 1984).

The maximum absorption (Soret maximum) of the carbon monoxide-reduced difference spectrum provides additional evidence of cytochrome P-450 induction. 3-Methylcholanthrene-type of inducers in both rats and chickens shift the absorbance maximum from the 450 nm peak seen in controls to a peak at 448 nm (Ronis and Walker, 1989). β -Naphthoflavone treatment of

chickens and rats in our study shifted the absorbance maximum by 2 nm in agreement with literature reports. Sesbania drummondii treatment did not shift the maximal absorption in either the chicken or the rat, which clearly distinguished it from 3-methylcholanthrene-type of induction. However, no other useful conclusions about patterns of change consistent with any other cytochrome P-450 isozyme can be drawn from this data. Identical absorption maxima have been obtained by several investigators for several of the cytochrome P-450 isozymes (Ryan and Levin, 1990).

The six cytochrome P-450-mediated activities characterized for comparisons of the catalytic properties of the hepatic cytochrome P-450 monooxygenase systems of the treated groups differed in their selectivity and specificity for the major inducible forms of cytochrome P-450. Three of the activities (aminopyrine N-demethylase, aldrin epoxidase, ethoxyresorufin O-deethylase) were evaluated because they yielded interesting data in the preliminary study. The three other activities (methoxy-, pentoxy-, and benzyloxyphenoxazone O-dealkylase) were studied because of their high specificity for cytochrome P-450 isozymes.

Aminopyrine N-demethylase in the rat hepatic microsome has been associated with the phenobarbital-inducible form of cytochrome P-450 (Lubet et al., 1985). Our results in the rat

and chicken agreed with this finding. Phenobarbital treatment induced this activity in both species. However, aminopyrine N-demethylase activity has limited utility because the induced rates of this activity are small (only 2.5 to 3-fold higher than uninduced controls) and the activity is not considered to be highly specific for the phenobarbital-inducible cytochrome P-450 (Lubet et al., 1985; Ronis and Walker, 1989). Guengerich et al. (1982) reported that aminopyrine N-demethylase was expressed by six of eight purified rat cytochrome P-450 forms, some inducible and some constitutive. In our experiment, the aminopyrine N-demethylase activity was increased in rats with phenobarbital treatment and in chickens with phenobarbital and β -naphthoflavone treatment. Therefore, we could distinguish between these two inducers in the rat but not the chicken with this activity. Ronis and Walker (1989) concluded that aminopyrine was a relatively poor substrate for avian monooxygenases because the aminopyrine N-demethylase activity in avian hepatic microsomes was only 25% of the corresponding activity in the rat hepatic microsomes. Our data in control chicken hepatic microsomes vs. rat hepatic microsomes for this activity disagrees with their conclusion. We observed virtually the same specific activity in both species.

Treatment with Sesbania drummondii altered the aminopyrine N-demethylase specific activity in both species. In the rat

treated with S. drummondii, the specific activity was much smaller than observed in control, phenobarbital, and β -naphthoflavone-treated animals. In the chicken, the specific activity was increased with S. drummondii treatment to a level of activity similar to β -naphthoflavone with the 0.5% dosage and then to an activity similar to phenobarbital for the 0.75% dosage treatment. These data suggest two points. First, the increase in the specific activity with increasing dosage of S. drummondii indicate that a dose-response relationship between S. drummondii treatment and the activity may exist. The second observation is that this activity is not useful in distinguishing which of the cytochrome P-450 forms might be induced by Sesbania drummondii. Comparisons of the molar activity of aminopyrine N-demethylase in the chicken confirm the lack of utility of this cytochrome P-450-mediated activity.

The aldrin epoxidase assay has also been associated with the phenobarbital-inducible form of cytochrome P-450 but with greater specificity (Lubet et al., 1985). We found this to be true in both chickens and rats. Phenobarbital treatment markedly increases aldrin epoxidase specific activity in both species, whereas, β -naphthoflavone treatment did not alter the specific activity in the chicken and actually induced a decrease in the rat specific activity. Recently, Wolff and Guengerich (1987) have questioned the specificity of the

aldrin epoxidase assay in rats. They found that seven purified cytochrome P-450 isozymes characteristic for the male and phenobarbital-treated rat catalyzed aldrin epoxidation. This work did confirm that aldrin epoxidation was not related to the cytochrome p-448 isozyme family.

One advantage to the aldrin epoxidase assay in the rat and the chicken is that the activity is induced higher by phenobarbital over the activity in uninduced controls than the corresponding induction of aminopyrine N-demethylase activity. Despite this advantage, the aldrin epoxidase assay has additional limitations in the avian cytochrome P-450 system. Aldrin is considered to be a relatively poor, non-specific substrate in birds (Ronis and Walker, 1989). Our data in uninduced chickens versus rats support the contention that aldrin is a poor substrate in birds as chickens exhibited only 30% of the activity observed in rats.

Sesbania drummondii treatment induced changes in aldrin epoxidation in both chickens and rats. A pronounced increase in aldrin epoxidase activity (3 to 4-fold) and molar activity was observed in S. drummondii-treated chickens. The magnitude of this induction was similar to phenobarbital with the 0.75% treatment group. This suggests that aldrin is equally good as a substrate for the cytochrome P-450 forms induced by either S. drummondii or phenobarbital in the chicken.

Rats treated with S. drummondii showed a significant 2-fold decrease in aldrin epoxidase specific activity. This 50% depression in activity was virtually identical to the depression in activity induced by β -naphthoflavone. Wolff and Guengerich (1987) have previously reported that β -naphthoflavone decreases aldrin epoxidation in rat hepatic microsomes. The reason the rat hepatic microsomes from animals treated with S. drummondii and β -naphthoflavone do not catalyze the epoxidation of aldrin as well as uninduced control animals is not known. Perhaps, these treatments alter the geometry of the active site of the cytochrome P-450 enzyme such that aldrin is not effectively bound and thus catalyzed. It is also unclear whether S. drummondii and β -naphthoflavone induce similar molecular changes that result in the decreased activity.

Presently, the O-dealkylation of various alkoxyphenoxazone derivatives are the most specific of the cytochrome P-450-mediated activities for distinguishing between inducible cytochrome P-450 isozymes in rat hepatic microsomes. Marked induction of the O-dealkylation of pentoxy- and benzyloxyphenoxazone is associated with the major phenobarbital-inducible isozyme in the rat, cytochrome P-450 IIB1 (Burke and Mayer, 1983; Burke et al., 1985). Marked induction of the O-dealkylation of ethoxyphenoxazone (ethoxyresorufin) is associated with the major 3-

methylcholanthrene-inducible isozyme in the rat, cytochrome P-450 IA1 (Burke and Mayer, 1983; Burke et al., 1985).

The induction pattern of liver microsomal alkoxyphenoxazone O-dealkylases has also proved useful in studying liver microsomes of some nonmammalian species e.g., the channel catfish (Ankley et al., 1987; Winston et al., 1989) and the alligator (Jewell et al., 1989). The utility of the hepatic microsomal metabolism of the alkoxyphenoxazones as a probe for the different types of cytochrome P-450 inducers in the avian species has not been reported. Studies in birds have thus far been limited to the ethoxy-derivative of hydroxyphenoxazone (Sawyer et al., 1986; Carpenter et al., 1985). Only one of these studies used White Leghorn chickens, thus there is a very limited data base on the induction pattern of O-dealkylation in substituted alkoxyphenoxazones in avian species in general, and especially in the chicken.

In our study, we observed that the alkoxyphenoxazone O-dealkylase activities of chickens and rats were similar in uninduced animals but were markedly different after induction with phenobarbital and β -naphthoflavone. In uninduced chickens, the highest alkoxyphenoxazone O-dealkylase activity was with ethoxyphenoxazone as the substrate followed in decreasing order by methoxyphenoxazone, benzyloxyphenoxazone, and pentoxyphenoxazone. The rate of metabolism of the

alkoxyphenoxazones in the uninduced rat followed a pattern similar to that of chickens with ethoxyphenoxazone showing the fastest and pentoxyphenoxazone showing the slowest rate of metabolism. In contrast, in the rat, benzyloxyphenoxazone was metabolized faster than methoxyphenoxazone.

Phenobarbital treatment in the rat has been associated with a marked induction (> 30-fold) of the O-dealkylation of pentoxy- and benzyloxyphenoxazones (Burke and Mayer, 1983; Burke et al., 1985). In good agreement with the literature, we observed as much as a 200-fold increase in pentoxyphenoxazone O-dealkylation and a 100-fold increase in benzyloxyphenoxazone O-dealkylation. Much smaller increases occurred in the phenobarbital pretreated rat with ethoxyphenoxazone (4-fold) and methoxyphenoxazone (3-fold). In contrast, we observed in the phenobarbital pretreated chicken similar small increases in the metabolism of ethoxyphenoxazone (1.5-fold) and methoxyphenoxazone but opposite effects (2-fold decreases) with pentoxyphenoxazone and benzyloxyphenoxazone. Therefore, in marked contrast to the rat enzymatic system, none of these alkoxyphenoxazone O-dealkylase activities appear to be significantly induced in the phenobarbital pretreated chicken. Perhaps other derivatives of phenoxazone will prove to be more useful in phenobarbital treated birds. To the best of our knowledge, only ethoxyphenoxazone O-dealkylase has been characterized and found to be unchanged in phenobarbital

pretreated birds (Carpenter et al., 1984 and 1985). Why phenobarbital pretreatment in chickens failed to induce either the O-dealkylation of pentoxy- or benzyloxyphenoxazone is not presently known. Two possibilities are that 1) phenobarbital induction induces a gene subfamily in the chicken different from P-450IIB1, the isozyme in the rat that is selective for pentoxy- and benzyloxyphenoxazone or 2) different regulatory mechanisms of induction exist between the two species.

Treatment with the 3-methylcholanthrene-type of cytochrome P-450 inducers in the rat has been associated with a marked induction (> 30-fold) of ethoxyphenoxazone O-dealkylase activity (Burke and Mayer, 1983; Burke et al., 1985). Burke et al. (1985) reported inductions in the metabolism of ethoxyphenoxazone of 51-fold with 3-methylcholanthrene and 74-fold with β -naphthoflavone in Sprague-Dawley rats. Matsubara et al. (1983) observed a smaller increase of 12-fold in male Wistar rats treated with β -naphthoflavone. In our investigation, we observed a nearly 20-fold induction of the O-dealkylation of ethoxyphenoxazone in rats pretreated with β -naphthoflavone. Much smaller increases of 9-fold with methoxyphenoxazone, 5-fold with pentoxyphenoxazone, and 6-fold with benzyloxyphenoxazone O-dealkylation were observed in our β -naphthoflavone treated rats. The degree of induction of the O-dealkylation of ethoxyphenoxazone in our β -naphthoflavone-treated chickens was similar in magnitude (27-fold) to the

rats. Sawyer *et al.* (1986) reported an even higher degree of induction of ethoxyphenoxazone O-dealkylation (37-fold) in 3-methylcholanthrene-treated White Leghorn cockerels. Carpenter *et al.* (1985) reported that β -naphthoflavone treatment resulted in a 13-fold induction of ethoxyphenoxazone O-dealkylase in Japanese quail. However, an even greater induction of benzyloxyphenoxazone O-dealkylase in White Leghorn chickens pretreated with β -naphthoflavone was observed in our study. β -Naphthoflavone treatment of chickens also produced higher degrees of induction in the metabolism of methoxyphenoxazone (14-fold) and pentoxyphenoxazone (9-fold) than was observed in the rat. Therefore, it appears that the O-dealkylation of benzyloxyphenoxazone and ethoxyphenoxazone are useful probes for studying β -naphthoflavone induction in the chicken.

As might be expected, the mixed-type of cytochrome P-450 inducers in rats combine many of the inducing characteristics of both phenobarbital and 3-methylcholanthrene on the O-dealkylation of the alkoxyphenoxazones. Similar to phenobarbital treatment in the rat, Aroclor 1254 treatment induces the metabolism of pentoxy- and benzyloxyphenoxazone. However, Burke *et al.* (1985) reported that Aroclor was much less effective than phenobarbital at inducing either depropylation (22-fold compared to 283-fold by phenobarbital) or debenzilation (30-fold compared to 95-fold by

phenobarbital). Other authors have observed higher levels of induction in these two activities with Aroclor. Lubet et al. (1985) observed that Aroclor 1254 increased the metabolism of pentoxyphenoxazone by 52-fold compared to a 94-fold increase with phenobarbital. Winston and Narayan (1990) also observed that Aroclor 1254 induced pentoxyphenoxazone O-dealkylation 50% as effectively as phenobarbital (38-fold compared to 75-fold by phenobarbital) in agreement with Lubet et al., (1985). However, in contrast to Burke et al. (1985), Winston and Narayan (1990) observed that Aroclor 1254 actually induced the metabolism of benzyloxyphenoxazone (60-fold) higher than that of phenobarbital treatment (25-fold). This suggests that benzyloxyphenoxazone may be a more specific probe for mixed-type rather than phenobarbital-type inducers in the rat. Whether this will also be true for avian species treated with Aroclor is not presently known.

In addition, in the rat Aroclor 1254 induces ethoxyphenoxazone O-dealkylation in a manner similar to that of 3-methylcholanthrene. Burke et al. (1985) observed the rate of the O-deethylation reaction to be virtually the same after Aroclor treatment (61-fold) as after induction by 3-methylcholanthrene (51-fold).

To the best our knowledge, in the chicken treated with mixed-type inducers only ethoxyphenoxazone O-dealkylation has been

characterized. Rifkind et al. (1984) observed a marked 50-fold induction in chicken embryos treated with 4 polychlorinated biphenyl congeners.

The O-dealkylation of the alkoxyphenoxazones has been evaluated in rats treated with a number of nonclassical cytochrome P-450 inducers including isosafrole, SKF-525A, pregnenolone 16 α -carbonitrile, imidazole N-substituted antifungal agents, and ethanol (Burke et al., 1985; Rodriques, 1987; Winston and Narayan, 1990). The changes induced by these compounds are variable and generally not as large or specific as those induced by the classical types of inducers. However, one distinction noted between the classical and nonclassical inducers is that phenobarbital, 3-methylcholanthrene, β -naphthoflavone, and Aroclor all stimulate metabolism of methoxyphenoxazone at appreciably higher rates than in controls, whereas, pregnenolone 16 α -carbonitrile, isosafrole, SKF 525A, and ethanol stimulate metabolism of methoxyphenoxazone at virtually control rates (Burke et al., 1985; Winston and Narayan, 1990).

Comparison of the effects of Sesbania drummondii treatment of chickens on the metabolism of pentoxy- and benzyloxyphenoxazones do not verify association with phenobarbital inducible isozymes because these 2 activities are not strongly induced in phenobarbital treated chickens as

they are in rats. However, there was a similar pattern in the metabolism of pentoxyphenoxazone by microsomes from chickens treated with S. drummondii at 0.75% of body weight as those from chickens treated with phenobarbital. Both treatments significantly decreased the specific activity compared to controls. However, the S. drummondii treated chickens differed from the phenobarbital treatment for the metabolism of benzyloxyphenoxazone. Phenobarbital treatment in chickens again depressed this activity but S. drummondii treatment had no effect. No differences between the above changes in these activities were observed for specific vs. molar activity in the chicken.

The Sesbania drummondii-treated rat was clearly different from phenobarbital-treated rats based on the pentoxyphenoxazone and benzyloxyphenoxazone O-dealkylase specific activity. Marked increases in the specific activities of both substrates occurred with phenobarbital, whereas, marked decreases occurred with S. drummondii treatment. This distinction was also present for the metabolism of pentoxyphenoxazone in terms of molar activity but absent for the molar activity of benzyloxyphenoxazone. This suggests that some of the differences induced by S. drummondii treatment in the rat are due to the decrease in the total concentration of cytochrome P-450.

Chickens treated with Sesbania drummondii were clearly different from β -naphthoflavone-treated birds with regard to the 3-methylcholanthrene-associated alkoxyphenoxazones. β -Naphthoflavone treatment markedly increased the metabolism of ethoxy-, pentoxy-, and benzyloxyphenoxazone, whereas S. drummondii treatment caused no apparent induction of these specific activities. β -Naphthoflavone and S. drummondii also showed no similarities in alkoxyphenoxazone O-dealkylation when they were compared in terms of molar activities. This observation was much different from that of the preliminary study in which we observed a marked induction of ethoxyresorufin O-dealkylase in chickens treated with S. drummondii. The reason for this discrepancy between our two studies is not known. The two studies had differences in the year that the S. drummondii crop was harvested, the methods of the assay, and in the laboratory and personnel where the assay was performed.

Sesbania drummondii-treated rats exhibited ethoxyphenoxazone O-dealkylation specific activity virtually the same as uninduced rats. In contrast, the β -naphthoflavone-treated rat exhibited a marked increase in the metabolism of ethoxyphenoxazone. The molar activities between these two groups were also different.

In the chicken, only the methoxyphenoxazone O-dealkylase

specific activity of S. drummondii-treated birds was significantly greater than controls. This increase was slightly greater than that induced by phenobarbital but was much less than that induced by β -naphthoflavone. This difference was not as evident for molar activities. The metabolism of methoxyphenoxazone in terms of the nmole of cytochrome P-450 present in the reaction was similar for control, S. drummondii, and phenobarbital treatments. The Sesbania drummondii-treated rat again exhibited specific and molar methoxyphenoxazone activities different from control and induced treatments.

A few observations are evident from the above comparisons of alkoxyphenoxazone O-dealkylation of Sesbania drummondii vs. phenobarbital and β -naphthoflavone treatments of chickens and rats. The treatment effects of S. drummondii in the rat were clearly different from the effects of phenobarbital and β -naphthoflavone on the alkoxyphenoxazones O-dealkylation. The S. drummondii treatment effects of chickens were definitely different from the β -naphthoflavone treatment. But unfortunately because one or more of the alkoxyphenoxazone O-dealkylases are not specifically induced by phenobarbital in the chicken, we can not clearly compare S. drummondii to phenobarbital with these activities. However, in at least one activity, pentoxyphenoxazone O-dealkylase, both S. drummondii and phenobarbital induced similar changes.

C. PHASE III - EFFECTS OF SESBANIA DRUMMONDII ON HEPATIC NADPH-DEPENDENT CYTOCHROME P-450 REDUCTASE. COMPARISON WITH EFFECTS CAUSED BY PHENOBARBITAL AND β -NAPHTHOFLAVONE.

OBJECTIVES:

1. To compare the effects of Sesbania drummondii, phenobarbital, and β -naphthoflavone on chicken and rat hepatic microsomal NADH- and NADPH-cytochrome c reductase activities.
2. To determine the NADPH-dependent cytochrome P-450 reductase contribution to the Sesbania drummondii-, phenobarbital-, and β -naphthoflavone-induced changes in hepatic microsomal monooxygenase activities by comparing NADPH-dependent metabolism to organic hydroperoxide-mediated (NADPH-independent) metabolism of aminopyrine and aldrin.

MATERIALS AND METHODS:

A. Chemicals:

4-Aminophenol (99% pure), β -naphthoflavone (5,6-benzoflavone, 90-95% pure), cumene hydroperoxide (80% w/v), and tert-butyl hydroperoxide (70 % w/v) were obtained from Sigma Chemical Co. (St. Louis, MO). Aldrin (99% pure) and dieldrin (98% pure) were obtained from Alltech Associates, Inc. (Deerfield, IL). t-Chlordane was obtained from the EPA (Research Triangle Park,

NC). Phenobarbital, sodium injection, (65 mg/mL) was obtained from Elkins-Sinn, Inc. (Cherry Hill, NJ). All other chemicals and biochemicals were of research grade or better and were obtained from various commercial sources.

B. Animals:

Male White Leghorn chickens and Sprague-Dawley rats were obtained, cared for, and assigned to treatment groups as described in detail in Phase II.

C. Treatment:

Animals were treated with either bicarbonate-azide buffer (controls), *S. drummondii* extract, phenobarbital, or β -naphthoflavone as described in detail in Phase II.

D. Preparation of hepatic microsomes:

Microsomes were prepared from pooled livers of the subgroups, treated or control, as described in detail in Experiment 1, Phase I.

E. Protein determination:

The concentration of protein of the microsomes was determined as described in detail in Experiment 1, Phase I.

F. Cytochrome P-450:

The cytochrome P-450 content of the microsomes was measured as

described in detail in Experiment 1, Phase I.

G. NADH- and NADPH-cytochrome c reductase:

NADH- and NADPH-cytochrome c reductase activities were assayed as described in detail in Experiment 1, Phase I.

H. Hepatic microsomal monooxygenase activities:

1. NADPH-dependent metabolism:

a. Aminopyrine N-demethylase:

The microsomal-mediated metabolism of aminopyrine was determined as described in detail in Phase I, Experiment 1 with the modifications described in Phase II. An NADPH-generating system was used to initiate this reaction.

b. Aldrin epoxidase:

The microsomal-mediated metabolism of aldrin was measured as described in detail in Phase I, Experiment 1 with the modifications described in Phase II. An NADPH-generating system was a component of the reaction mixture for this assay.

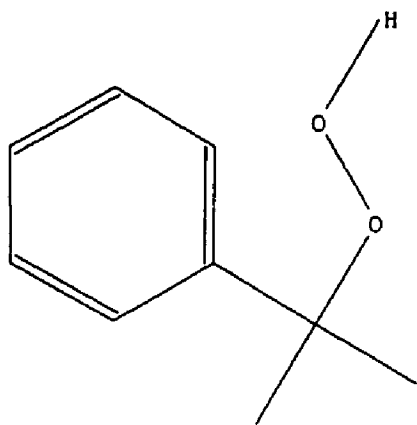
2. Organic hydroperoxide-mediated metabolism :

a. Aminopyrine N-demethylase:

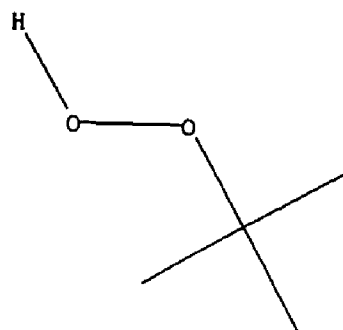
1) Cumene hydroperoxide: The assay for the cumene hydroperoxide-mediated metabolism of aminopyrine was similar to the NADPH-dependent assay detailed in Phase I, Experiment 1 and Phase II except for a few modifications. The first

change was the elimination of the NADPH generating system (NADP⁺, glucose 6-phosphate, and glucose 6-phosphate dehydrogenase) from the reaction mixture and replacing it with cumene hydroperoxide (Figure 8). A fresh stock suspension of cumene hydroperoxide (0.15 M in potassium phosphate buffer, 1.0 M, pH 7.4) was added to the reaction mixture to start the reaction. The final concentration of cumene hydroperoxide in the reaction mixture was 0.5 mM (Winston and Cederbaum, 1982 and 1983b). The microsomal protein concentration and the incubation time were changed in the assay to optimize the conditions for linearity. Appendices 23-24 illustrate the optimum conditions for the cumene hydroperoxide-mediated metabolism of aminopyrine in control chicken and rat hepatic microsomes. These conditions (Table 20) were utilized in determining the cumene hydroperoxide-mediated aminopyrine N-demethylase activity of control, S. drummondii-, phenobarbital, and β -naphthoflavone-treated animals.

2) t-Butyl hydroperoxide: The assay for the t-butyl hydroperoxide-mediated metabolism of aminopyrine was very similar to the cumene hydroperoxide-mediated assay described above. The reaction, however, was initiated by adding t-butylhydroperoxide (0.15 M stock solution in potassium phosphate buffer, 1.0 M, pH 7.4) (Figure 8). The final concentration of t-butyl hydroperoxide in the reaction mixture was 0.5 mM (Winston and Cederbaum, 1982 and 1983b). Optimal



Cumene hydroperoxide



t-Butyl hydroperoxide

Figure 8. Chemical structures of the organic hydroperoxides.

Table 20: Optimized conditions for the assay of cumene hydroperoxide-mediated aminopyrine N-demethylase activity in hepatic microsomes from control chickens and rats

Condition	Chicken	Rat
Time (min)	10	10
Protein concentration (mg/mL)	1.0	1.0
Substrate concentration (mM)	10.0	10.0

All assays were performed at 37°C. The assays were conducted in duplicate using a single control chicken or rat hepatic microsomal preparation over a range of 3 protein concentrations, 5 incubation times, and 6 concentrations of the aminopyrine substrate. The protein concentrations and incubation times yielded maximal product formation in the linear range (see Appendices 23-24).

assay conditions with respect to microsomal protein concentration and incubation time were determined using t-butyl hydroperoxide with control chicken and rat hepatic microsomes (Appendices 25-26). These conditions (Table 21) were utilized in measuring the t-butyl hydroperoxide-mediated aminopyrine N-demethylase activities in control, S. drummondii-, phenobarbital, and β -naphthoflavone-treated chickens and rats.

b. Aldrin epoxidase:

1. Cumene hydroperoxide: The assay for the cumene hydroperoxide-mediated metabolism of aldrin was similar to the NADPH-dependent method detailed in Phase I, Experiment 1 and Phase II except for a few modifications. The first change involved substituting cumene hydroperoxide for the NADPH generating system. A stock cumene hydroperoxide suspension (0.0525 M) was freshly prepared by adding the concentrate to potassium phosphate buffer solution (0.25 M, pH 6.8). This suspension (0.5 mM final concentration in the reaction mixture) was used instead of aldrin to initiate the reaction. The concentration of cumene hydroperoxide selected for the aldrin epoxidase assay was the same concentration used by Winston and Cederbaum (1982,1983b) with the aminopyrine N-demethylase assay.

Table 21: Optimized conditions for the assay of t-butyl hydroperoxide-mediated aminopyrine N-demethylase activity in hepatic microsomes from control chickens and rats

Condition	Chicken	Rat
Time (min)	15	15
Protein concentration (mg/mL)	2.0	2.0

Assays were performed at 37°C using 10.0 mM aminopyrine substrate concentration. The assays were conducted over a range of 3 protein concentrations and 3 incubation times using a single control chicken or rat hepatic microsomal preparation. The protein concentrations and incubation times yielded maximal product formation in the linear range (see Appendices 25-26).

To the best of our knowledge, the organic hydroperoxide-mediated metabolism of aldrin has not been previously reported with rat or chicken hepatic microsomes. Additional changes in the microsomal protein concentration and incubation time were necessary to assure maximal linear velocity of the reaction. A linear range for protein concentration and time were determined for control chicken and rat hepatic microsomes (Appendices 27-28). These conditions (Table 22) were utilized in measuring the cumene hydroperoxide-mediated metabolism of aldrin in control, *S. drummondii*-, phenobarbital, and β -naphthoflavone-treated chickens and rats. It was also necessary to reduce the extraction volume of 2,2,4-trimethylpentane from 5 mL to 2 mL in order to measure the smaller concentrations of dieldrin produced.

2. t-Butyl hydroperoxide: The assay for the t-butyl hydroperoxide-mediated metabolism of aldrin was essentially the same as described above except that t-butyl hydroperoxide was used instead of cumene hydroperoxide. A freshly prepared stock solution (0.05 M in 0.25 M potassium phosphate buffer, pH 6.8) was added to the reaction mixture to start the reaction. The final concentration of t-butyl hydroperoxide in the reaction mixture was 0.5 mM. Again, changes in the microsomal protein concentration and in the incubation time were necessary in order to achieve maximal linear reaction velocities. A linear range of protein concentration and

Table 22: Optimized conditions for the assay of cumene hydroperoxide-mediated aldrin epoxidase activity in hepatic microsomes from control chickens and rats

Condition	Chicken	Rat
Time (min)	2	2
Protein concentration (mg/mL)	0.4	0.2
Substrate concentration (μ M)	50.0	50.0

All assay were performed at 37°C. The assays were conducted in duplicate using a single control chicken or rat hepatic microsomal preparation over a range of 3 protein concentrations, 5-6 incubation times, and 5 concentrations of aldrin substrate. The protein concentrations and incubation times yielded maximal product formation in the linear range (see Appendices 27-28).

incubation time were determined with control chicken and rat hepatic microsomes (Appendices 29-30). These optimal conditions (Table 23) were used in measuring the t-butyl hydroperoxide-mediated metabolism of aldrin in control, S. drummondii-, phenobarbital, and β -naphthoflavone-treated chickens and rats.

Table 23: Optimized conditions for the assay of t-butyl hydroperoxide-mediated aldrin epoxidase activity in hepatic microsomes from control chickens and rats

Condition	Chicken	Rat
Time (min)	10	5
Protein concentration (mg/mL)	0.5	0.4

Assays were performed at 37°C using 50 μ M aldrin substrate concentration. The assays were conducted in duplicate using a single control chicken or rat hepatic microsomal preparation over a range of 3 protein concentrations and 6 incubation times. The protein concentrations and incubation times yielded maximal product formation in the linear range (see Appendices 29-30).

I. Statistical Analyses:

Statistical analyses of the data were performed using an analysis of variance (ANOVA) among the chicken or rat experimental groups. This was accomplished by determining the mean and standard deviation for each group. Differences among the means and standard deviations were tested for significance using Scheffe's multiple range test (Snedecor and Cochran, 1980). These calculations were carried out on an IBM-PC/AT computer (IBM, Dallas, TX) using the STATGRAPHICS^R program (version 2.1) by Statistical Graphics Corp. (Rockville, MD). The acceptable level of significance for this study was established at $P < 0.05$.

RESULTS:**NADH-and NADPH-cytochrome c reductase:**

The effect of Sesbania drummondii treatment on NADH- and NADPH-cytochrome c reductase activities in chicken and rat hepatic microsomes (Table 24) corroborated the findings of the preliminary study (Table 2). Significant differences were not observed for NADH-cytochrome c reductase activity in either species treated with Sesbania drummondii. The NADPH-cytochrome c reductase activity was significantly increased with S. drummondii treatment of chickens (0.75%) but unchanged in the treated-rats. Treatment with phenobarbital stimulated changes in rat but not chicken reductase activity. A significant decrease in rat and no change in chicken NADH-cytochrome c reductase activity occurred with phenobarbital treatment. Likewise, NADPH-cytochrome c reductase activity in the chicken was unaffected by phenobarbital. A change of NADPH-cytochrome c reductase activity was observed in the phenobarbital-treated rat but, instead of a decrease, a significant increase in activity occurred. β -Naphthoflavone treatment had no effect on either NADH- or NADPH-cytochrome c reductase activities of either chicken and rat hepatic microsomes.

Table 24: The effect of *Sesbania drummondii*, phenobarbital, and β -naphthoflavone on chicken and rat hepatic NADH- and NADPH-cytochrome c reductase activities.

Treatment	Cytochrome c reductase	
	NADH-	NADPH-
Chicken		
Control	159 \pm 22 ^a	84 \pm 11 ^{ab}
<i>Sesbania</i> (0.5% BW)	166 \pm 41 ^a	122 \pm 23 ^b
<i>Sesbania</i> (0.75% BW)	166 \pm 18 ^a	176 \pm 31 ^c
Phenobarbital	138 \pm 28 ^a	82 \pm 13 ^a
β -Naphthoflavone	163 \pm 24 ^a	84 \pm 19 ^{ab}
Rat		
Control	606 \pm 132 ^a	151 \pm 15 ^a
<i>Sesbania</i> (0.5% BW)	538 \pm 50 ^a	145 \pm 22 ^a
Phenobarbital	344 \pm 20 ^b	235 \pm 9 ^b
β -Naphthoflavone	646 \pm 32 ^a	131 \pm 6 ^a

Values are the mean \pm SEM, n = 6.

Reductase activity expressed as nmole cytochrome c reduced / min / mg protein.

Means with like superscripts within the same species are not significantly different (P > 0.05).

Hepatic microsomal monooxygenase activities:NADPH-dependent metabolism:Aminopyrine N-demethylase:

The effect of Sesbania drummondii, phenobarbital, and β -naphthoflavone on NADPH-dependent aminopyrine N-demethylase activity in chicken and rat hepatic microsomes (Table 14) is described in detail in Phase II.

Aldrin epoxidase:

The effect of Sesbania drummondii, phenobarbital, and β -naphthoflavone on NADPH-dependent aldrin epoxidase activity of chicken and rat hepatic microsomes (Table 15) is described in detail in Phase II.

Organic hydroperoxide-mediated metabolism:Aminopyrine N-demethylase:

Cumene hydroperoxide: Species differences were evident in the capability of cumene hydroperoxide to support the N-demethylation of aminopyrine (Table 25). Cumene hydroperoxide appeared to more effectively mediate aminopyrine N-demethylation in rat hepatic microsomes resulting in specific activities nearly 2-fold greater than the corresponding specific activity in the NADPH-dependent reaction. In contrast, the chicken hepatic microsomal fraction was less effective in catalyzing the aminopyrine N-demethylation with cumene hydroperoxide as the oxygen and electron donor. The

Table 25: The effect of *Sesbania drummondii*, phenobarbital, and β -naphthoflavone on cumene hydroperoxide-mediated aminopyrine N-demethylase activity in hepatic microsomes from chickens and rats

Treatment	nmoles Formaldehyde formed / min	
	per mg protein	per nmole P-450
Chicken		
Control	1.35 ± 0.17^a	8.12 ± 0.72^{ab}
<i>Sesbania</i> (0.5% BW)	2.82 ± 0.48^b	8.62 ± 1.33^a
<i>Sesbania</i> (0.75% BW)	3.02 ± 0.76^b	9.49 ± 1.29^a
Phenobarbital	2.33 ± 0.26^b	6.52 ± 0.85^b
β -Naphthoflavone	2.39 ± 0.31^b	3.82 ± 0.50^c
Rat		
Control	8.10 ± 0.91^a	9.48 ± 0.56^{ab}
<i>Sesbania</i> (0.5% BW)	4.79 ± 0.58^b	10.09 ± 0.91^b
Phenobarbital	17.98 ± 2.72^c	8.39 ± 0.65^a
β -Naphthoflavone	8.27 ± 0.89^a	6.80 ± 0.69^c

Values are the mean \pm SEM, n = 6.

Means with like superscripts within the same species are not significantly different (P > 0.05).

specific activities of the cumene hydroperoxide reactions were 50% (or less) of the corresponding NADPH-dependent mixed function oxidase reactions. Despite the quantitative differences in specific activity, the cumene hydroperoxide- and NADPH-mediated aminopyrine N-demethylase activities showed similar patterns of change as a function of pretreatment in each of the treated groups. The cumene hydroperoxide-mediated aminopyrine N-demethylase specific activities of chickens treated with Sesbania drummondii, phenobarbital, and β -naphthoflavone were all approximately 2-fold greater than that of the controls. However, unlike the NADPH-dependent reaction, no significant difference between the two chicken Sesbania drummondii treatment groups was observed in the cumene hydroperoxide-mediated reaction. The specific activities of the cumene hydroperoxide-mediated reactions of rats treated with Sesbania drummondii, phenobarbital, and β -naphthoflavone followed the identical pattern of change as that of the NADPH-dependent reaction. Sesbania drummondii treatment resulted in a decrease, phenobarbital an increase, and β -naphthoflavone, no change in the cumene hydroperoxide-mediated aminopyrine N-demethylase specific activity.

Similarities were also present between molar activities of the cumene hydroperoxide-mediated and NADPH-dependent N-demethylation of aminopyrine. In chickens, both dosage treatments with Sesbania drummondii were similar to controls and different from the phenobarbital and β -naphthoflavone molar activities. Sesbania drummondii treatment of rats resulted in molar activities statistically similar to control and phenobarbital-treated animals. The cumene hydroperoxide-mediated aminopyrine N-demethylase molar activity of animals treated with phenobarbital were not significantly different from control chicken and rat activities. In contrast, treatment with β -naphthoflavone resulted in a significant change in the cumene hydroperoxide-mediated molar activity of both species that was different from all other groups.

t-Butyl hydroperoxide: t-butyl hydroperoxide was less effective in mediating the N-demethylation of aminopyrine in both chicken and rat hepatic microsomes (Table 26). The specific activities of t-butyl hydroperoxide-mediated aminopyrine N-demethylation in both species, control and treated, were much lower than the corresponding activities using NADPH or cumene hydroperoxide. Treatment-induced changes in the specific activities also differed with t-butyl hydroperoxide. No change in specific activity was observed in chicken hepatic microsomes from any of the treatment groups. The t-butyl hydroperoxide-mediated aminopyrine

Table 26: The effect of *Sesbania drummondii*, phenobarbital, and β -naphthoflavone on t-butyl hydroperoxide-mediated aminopyrine N-demethylase activity in hepatic microsomes from chickens and rats.

Treatment	nmoles Formaldehyde formed / min	
	per mg protein	per nmole P-450
Chicken		
Control	0.46 ± 0.17^a	2.82 ± 0.98^a
<i>Sesbania</i> (0.5% BW)	0.49 ± 0.13^a	1.52 ± 0.42^b
<i>Sesbania</i> (0.75% BW)	0.59 ± 0.20^a	1.84 ± 0.47^{ab}
Phenobarbital	0.41 ± 0.15^a	1.14 ± 0.44^b
β -Naphthoflavone	0.50 ± 0.24^a	0.80 ± 0.36^b
Rat		
Control	0.71 ± 0.11^a	0.83 ± 0.09^a
<i>Sesbania</i> (0.5% BW)	0.42 ± 0.05^b	0.88 ± 0.08^a
Phenobarbital	1.92 ± 0.28^c	0.90 ± 0.04^a
β -Naphthoflavone	0.94 ± 0.21^d	0.77 ± 0.17^a

Values are the mean \pm SEM, n = 6.

Means with like superscripts within the same species are not significantly different (P > 0.05).

N-demethylase specific activity of rats followed a similar pattern of change as observed with cumene hydroperoxide and NADPH but with one distinct difference, i.e., the changes in specific activity that were similar included a decrease with Sesbania drummondii treatment and an increase with phenobarbital treatment. However, with β -naphthoflavone treatment, the t-butyl hydroperoxide-mediated reaction was slightly, but significantly, increased. No change in activity was observed in the β -naphthoflavone treated rat with the NADPH and cumene hydroperoxide reaction systems.

Differences between treated groups were less evident when the t-butyl hydroperoxide-mediated aminopyrine N-demethylase activities were expressed per nmole of cytochrome P-450. For example, the molar activities of chickens treated with Sesbania drummondii, phenobarbital, and β -naphthoflavone were different from controls but not different from each other. No differences between the treated groups and controls were observed in the rat t-butyl hydroperoxide-mediated molar activities.

Aldrin epoxidase:

Cumene hydroperoxide: Species differences were apparent in the capability of cumene hydroperoxide to mediate the epoxidation of aldrin (Table 27). Cumene hydroperoxide was able to mediate aldrin epoxidation as effectively as NADPH in rat hepatic microsomes. This was suggested by the similar aldrin epoxidase specific activities in rat control hepatic microsomes utilizing NADPH and cumene hydroperoxide. In contrast, cumene hydroperoxide was much less effective in mediating aldrin epoxidation in the chicken hepatic microsome. The cumene hydroperoxide-mediated aldrin epoxidase specific activity of control chicken microsomes was only 25% of the NADPH-dependent specific activity.

Despite the quantitative differences in specific activity between the two systems in the chicken, the pattern of change in activity induced by the various treatment regimens was similar. Treatment with Sesbania drummondii and phenobarbital resulted in a similar increase in specific activity significantly greater than controls. However, phenobarbital treatment did not significantly increase the activity over that of Sesbania drummondii-treatment as had been observed with the NADPH reaction. Treatment with β -naphthoflavone resulted in no change in the specific activity of the cumene hydroperoxide-mediated aldrin epoxidase reaction.

Table 27: The effect of Sesbania drummondii, phenobarbital, and β -naphthoflavone on cumene hydroperoxide-mediated aldrin epoxidase activity in hepatic microsomes from chickens and rats.

Treatment	nmoles Dieldrin produced / min	
	per mg protein	per nmole P-450
Chicken		
Control	0.42 ± 0.05^a	2.50 ± 0.29^a
<u>Sesbania</u> (0.5% BW)	1.82 ± 0.44^b	5.53 ± 0.99^b
<u>Sesbania</u> (0.75% BW)	2.19 ± 0.57^b	6.88 ± 0.99^b
Phenobarbital	2.12 ± 0.39^b	5.89 ± 1.02^b
β -Naphthoflavone	0.42 ± 0.06^a	0.67 ± 0.09^c
Rat		
Control	4.07 ± 0.31^{ab}	4.78 ± 0.35^a
<u>Sesbania</u> (0.5% BW)	1.66 ± 0.25^c	3.49 ± 0.43^b
Phenobarbital	4.59 ± 0.56^b	2.15 ± 0.17^c
β -Naphthoflavone	3.75 ± 0.33^a	3.08 ± 0.17^b

Values are the mean \pm SEM, n = 6.

Means with like superscripts within the same species are not significantly different ($P > 0.05$).

The specific activity of the cumene hydroperoxide-mediated aldrin epoxidase of rat hepatic microsomes followed a pattern of changes similar to the NADPH-dependent reaction with one distinct difference. The effect of Sesbania drummondii and β -naphthoflavone treatments on the cumene hydroperoxide-mediated epoxidation of aldrin coincided with the changes in specific activity observed in the NADPH-dependent reaction. Sesbania drummondii treatment resulted in a 2-fold decrease and β -naphthoflavone resulted in no change of the specific activity of aldrin epoxidase. The effect of treatment with phenobarbital differed markedly between the two reaction systems. Phenobarbital treatment elevated the NADPH-dependent reaction 5-fold but did not induce a change in the cumene hydroperoxide-mediated reaction system.

The changes in cumene hydroperoxide-mediated aldrin epoxidase molar activity of both treated chickens and rats paralleled the activities of the NADPH-dependent reaction system. All 4 rat groups had molar activities that were distinctly different. Induced changes in molar activities of treated chickens distinguished the controls from Sesbania drummondii and phenobarbital, and from β -naphthoflavone treatment. Treatment with phenobarbital and Sesbania drummondii at 0.75% of body weight, however, were not significantly different from the 0.5% body weight Sesbania drummondii treatment.

t-Butyl hydroperoxide: t-butyl hydroperoxide was a poor mediator of aldrin epoxidation in both chicken and rat hepatic microsomes (Table 28). The specific activities of aldrin epoxidase were much lower in both species with the t-butyl hydroperoxidase-dependent than with the NADPH plus oxygen and cumene hydroperoxide-dependent reaction systems. The effect of treatments on t-butyl hydroperoxide-mediated aldrin epoxidase specific activity in both chickens and rats were similar to the effects observed with the cumene hydroperoxide-mediated reaction system. Treatment of chickens with Sesbania drummondii and phenobarbital resulted in an increase of similar magnitude in the specific activity whereas β -naphthoflavone treatment had no effect on activity. The effect of treatments on the rat t-butyl specific activities were distinctly different from the effects observed with cumene hydroperoxide and NADPH.

Sesbania drummondii treatment resulted in a slight but not significant drop in specific activity. Treatment with phenobarbital increased the specific activity 2-fold. No effect on the t-butyl hydroperoxide-mediated aldrin epoxidase activity was observed in rat hepatic microsomes from animals treated with β -naphthoflavone.

Table 28: The effect of *Sesbania drummondii*, phenobarbital, and 8-naphthoflavone on t-butyl hydroperoxide-mediated aldrin epoxidase activity in hepatic microsomes from chickens and rats.

Treatment	nmoles Dieldrin produced / min	
	per mg protein	per nmole P-450
Chicken		
Control	0.03 ± 0.01 ^a	0.19 ± 0.01 ^a
<i>Sesbania</i> (0.5% BW)	0.13 ± 0.03 ^b	0.41 ± 0.06 ^b
<i>Sesbania</i> (0.75% BW)	0.14 ± 0.04 ^b	0.44 ± 0.06 ^b
Phenobarbital	0.13 ± 0.03 ^b	0.37 ± 0.07 ^b
8-Naphthoflavone	0.04 ± 0.01 ^a	0.05 ± 0.01 ^c
Rat		
Control	0.80 ± 0.05 ^{ab}	0.94 ± 0.09 ^a
<i>Sesbania</i> (0.5% BW)	0.62 ± 0.10 ^a	1.30 ± 0.17 ^b
Phenobarbital	1.83 ± 0.21 ^c	0.86 ± 0.02 ^{ac}
8-Naphthoflavone	0.86 ± 0.12 ^b	0.71 ± 0.09 ^c

Values are the mean ± SEM, n = 6.

Means with like superscripts within the same species are not significantly different (P > 0.05).

The relative differences in the t-butyl hydroperoxide-mediated aldrin epoxidase molar activities of treated chickens exactly paralleled the changes observed in the cumene hydroperoxide-mediated reaction. The relative difference in molar activity in the rat treatment groups was unique to the t-butyl hydroperoxide-mediated reaction. Treatment with Sesbania drummondii altered the specific activity; however, treatment with phenobarbital did not significantly alter the molar activity. Treatments with phenobarbital and β -naphthoflavone in rat hepatic microsomes had overlapping t-butyl hydroperoxide-mediated aldrin epoxidase molar activities.

DISCUSSION:

The cytochrome P-450 monooxygenase system is a coupled enzyme system composed of two enzymes: cytochrome P-450 and NADPH cytochrome P-450 reductase (Sipes and Gandolfi, 1986). Accompanying this complex is cytochrome b_5 and its associated reductase, cytochrome b_5 reductase (Sipes and Gandolfi, 1986).

The cytochrome P-450 component of this system was found to be affected by treatments with an extract of Sesbania drummondii. Changes in the cytochrome P-450 specific content were observed in chickens and rats treated with Sesbania drummondii. The cytochrome b_5 specific content was unaffected by S. drummondii treatment in either species. The NADPH-cytochrome c reductase activity was increased in the chicken and unaffected in the rat by Sesbania drummondii treatment, whereas neither chickens nor rats demonstrated changes in NADH-cytochrome c reductase with treatment. Sesbania drummondii treatment was also observed to change several cytochrome P-450-mediated activities in both chickens and rats. However, because these enzyme activities are influenced by contributions of both the cytochrome P-450 and reductase components of the cytochrome P-450 monooxygenase system it is not possible to distinguish the relative contribution of changes in either component to the overall change in activity.

The purpose of the Phase III study was to evaluate the effect

of Sesbania drummondii on the reductase component of the hepatic cytochrome P-450 monooxygenase system. This was done by first comparing S. drummondii, phenobarbital, and β -naphthoflavone treatments on chicken and rat hepatic microsomal NADH- and NADPH-cytochrome c reductase activities.

NADH- and NADPH-cytochrome c reductase activities are an indirect measure of the reductase components of the cytochrome P-450 system. The cytochrome c reductase activities are not a measure of the reductase specific contents but instead measure the rate of electron transfer from the reductases.

None of the treatments altered the rate of electron transfer from NADH-cytochrome b_5 reductase in the chicken. And in the rat, only phenobarbital treatment altered the rate of electron transfer from this reductase by decreasing the reduction of cytochrome c. The significance of this finding as it relates to phenobarbital induction in the rat is not known.

Phenobarbital treatment in the rat had the opposite effect on the NADPH-cytochrome c reductase activity. In agreement with literature reports (Snyder and Remmer, 1979), an increase of 1.6-fold over the control rate was observed in the rate of electron transfer from NADPH cytochrome P-450 reductase in rats treated with phenobarbital. Neither Sesbania drummondii nor β -naphthoflavone treatment affected NADPH-cytochrome c

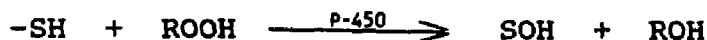
reductase activity in the rat. In contrast, chickens treated with phenobarbital did not induce NADPH-cytochrome c reductase activity. This finding disagreed with a report by Pilch and Combs (1981) that described a nearly 2-fold increase in NADPH-cytochrome c reductase activity in 12-day old cockerels given phenobarbital and fed diets supplemented with vitamin E and selenium. To the best of our knowledge, there are no other reports of the effect of phenobarbital treatment on chicken NADPH-cytochrome c reductase activity. Sesbania drummondii administered at a dosage of 0.75% of body weight to the chicken, was the only treatment that increased the NADPH-cytochrome c reductase activity. This increase was slightly lower than observed in the preliminary study. This difference may be due to a dose-related effect. Increases in activity of 1.5-fold with 0.5% treatment and 2.0-fold with 0.75% treatment were observed in this study, whereas a 2.5-fold increase was observed with treatment at 0.8% of body weight in the preliminary study.

From these observations it appears Sesbania drummondii increases the rate of electron transfer from the NADPH cytochrome P-450 reductase component of the cytochrome P-450 monooxygenase system in the chicken but not the rat. Sesbania drummondii does not change the rate of electron transfer from the NADH cytochrome b₅ reductase in either the chicken or the rat. However, it is not possible to determine from these data

what these findings represent in terms of the overall cytochrome P-450 monooxygenase system.

It is well established (White and Coon, 1980) that the NADPH cytochrome P-450 reductase is the rate limiting component of the cytochrome P-450 catalytic cycle. Therefore, changes induced in the rate of electron transfer may result in increased cytochrome P-450 enzyme activity. The contribution of the NADPH cytochrome P-450 reductase to the cytochrome P-450-mediated activity can be determined by comparing the NADPH-dependent metabolism to the organic hydroperoxide (NADPH-independent) metabolism of cytochrome P-450 substrates.

Organic hydroperoxides can replace NADPH and oxygen in supporting the metabolism of xenobiotics by the liver microsomal cytochrome P-450 system (Kadlubar *et al.*, 1973; Rahimluta and O'Brien, 1974). This type of activity reflects the ability of cytochrome P-450 to act as a peroxygenase as illustrated below (White and Coon, 1980):



The organic hydroperoxides have performed in a number of different hydroxylation reactions including: aliphatic (Nordblom and Coon, 1977) and aromatic hydroxylation (Rahimtula and O'Brien, 1974), N- or O-dealkylation (Nordblom

and Coon, 1977), alcohol dehydrogenation (Rahimtula and O'Brien, 1977) and alkene epoxidation (White and Coon, 1980).

Two cytochrome P-450-mediated activities, aminopyrine N-demethylase and aldrin epoxidase, were selected for comparisons of NADPH-dependent versus organic hydroperoxide-dependent metabolism in Sesbania drummondii, phenobarbital, and β -naphthoflavone-treated animals. Aminopyrine N-demethylase has been studied using both of these systems in the rat but not the chicken (Kadlubar et al., 1973; Winston and Cederbaum, 1983a and 1983b; Estabrook et al., 1984). To the best of our knowledge, aldrin epoxidase has not been previously reported utilizing organic hydroperoxides in either species.

In the Sesbania drummondii-treated chicken, increases in both the NADPH-dependent aminopyrine N-demethylase and aldrin epoxidase specific and molar activities were observed. Difference between these two activities, however, were evident for cumene hydroperoxide as the mediator. The specific activity of the cumene hydroperoxide-dependent metabolism of aminopyrine was similarly increased as the NADPH-dependent metabolism, whereas the molar activity was unchanged from the control rate with the cumene hydroperoxide-dependent reaction but increased in the NADPH-dependent reaction at the 0.75% dosage treatment with S. drummondii. This suggests that the

distinct difference in the NADPH-dependent aminopyrine N-demethylase activity in the chicken treated with S. drummondii may not be due only to a change in the cytochrome P-450 isozyme profile responsible for the metabolism of aminopyrine but may instead be due to the induction of the NADPH cytochrome P-450 reductase which when increased is no longer rate limiting resulting in a higher rate of activity. When this change is removed as was done by substituting the cumene hydroperoxide, the difference in the molar activity between control and S. drummondii-treated chickens is no longer observed. Similar to the results with S. drummondii, differences in aminopyrine N-demethylase molar activity were not observed with phenobarbital treatment of chickens. This finding is more difficult to explain in terms of the reductase component because phenobarbital in the chicken did not increase the rate of electron transfer from the NADPH cytochrome P-450 reductase. Perhaps instead, phenobarbital altered the cytochrome P-450 isozyme profile such that the vector arrangement between the reductase and the cytochrome P-450 isozymes was more efficient for electron transfer. The difference induced by β -naphthoflavone treatment in chickens was equally evident for both NADPH- and cumene hydroperoxide-dependent aminopyrine N-demethylase reaction suggesting that the increase in activity was truly due to induction of a specific cytochrome P-450 isozyme family.

In contrast to the aminopyrine N-demethylase activity in the chicken, aldrin epoxidation was induced in both NADPH and cumene hydroperoxide systems for both specific and molar activities with Sesbania drummondii treatment. This implies that S. drummondii induces the isozyme family responsible for the metabolism of aldrin because the induction of the reductase component does not contribute to the increased activity. This was also true for phenobarbital treatment. Aldrin epoxidase activity was increased with and without NADPH which also indicates that an isozyme family that metabolizes aldrin is induced by phenobarbital. The effect of β -naphthoflavone treatment on the metabolism of aldrin was similar in both NADPH and cumene hydroperoxide systems. The findings with phenobarbital and β -naphthoflavone were not unexpected since neither of these inducers were found to increase NADPH cytochrome P-450 reductase in the chicken.

In the rat, Sesbania drummondii treatment decreased both NADPH-dependent metabolism of aminopyrine and aldrin. We can conclude that this decrease is partially the result of a decrease in the cytochrome P-450 component because we observed a decrease in the cytochrome P-450 specific content as well as normalizing of the aminopyrine N-demethylase activity to the control rate by expressing the activity as per nmole of P-450. However, we did observe a specific decrease in the aldrin epoxidase molar activity which may indicate a specific change

in the constitutive isozyme profile. This finding may also be partly the result of S. drummondii induced faulty transfer of electrons from the reductase component to the cytochrome P-450 isozyme. This was the question addressed by using cumene hydroperoxide. We found that the cumene hydroperoxide-mediated aldrin epoxidase reaction followed a pattern similar to the NADPH-dependent reaction for both aminopyrine N-demethylation and aldrin epoxidation. This implies that in terms of aldrin epoxidation in the rat, Sesbania drummondii treatment does not appear to cause a faulty electron transfer because if it had then the cumene hydroperoxide-dependent activity would have approached the rate of the NADPH-dependent activity. Instead, it appears that S. drummondii treatment of the rat actually changed the isozyme profile such that aldrin is less effectively metabolized.

The contribution of the NADPH cytochrome P-450 reductase component of the cytochrome P-450-mediated metabolism of aminopyrine is clearly established by comparing cumene hydroperoxide-dependent metabolism to NADPH-dependent metabolism in phenobarbital-treated rats. Phenobarbital treatment increases both specific and molar activities of NADPH-dependent aminopyrine metabolism. However, with the cumene hydroperoxide-dependent metabolism, and when the rate of the reaction is adjusted for the total cytochrome P-450 content, the molar activity for the phenobarbital-treated rats

is equivalent to that of control rats. This suggests that the phenobarbital induction of the NADPH cytochrome P-450 reductase in rats result in an abundance of this component which is no longer rate limiting and the metabolism proceeds at a greater rate.

The relationship of the reductase component to aldrin epoxidation in the phenobarbital-treated rat was not clearly established using cumene hydroperoxide. In the NADPH-dependent epoxidation of aldrin, phenobarbital treatment resulted in a marked increase in activity. However, with the cumene hydroperoxide-dependent reaction the specific activity was not different from controls. But when adjusted for the elevation of total cytochrome P-450 specific content, the difference of phenobarbital treatment was evident. There are two possible explanations for this finding. Firstly, the increase in the aldrin epoxidase activity that occurs with phenobarbital treatment is related to the induction of the NADPH reductase component and not to specific changes in the cytochrome P-450 isozymes. The second explanation addresses mechanistic complications with cumene hydroperoxide. In addition to contributing electrons and oxygen, cumene hydroperoxide also is reported to interact with the binding domain of cytochrome P-450 (Rahimtula and O'Brien, 1975; O'Brien and Rahimtula, 1980). This phenomena is likely to effect particular isozymes of cytochrome P-450 more than

others and potentially block the ability of a substrate to interact with that cytochrome and be metabolized. This complication could explain our findings with cumene hydroperoxide and aldrin in phenobarbital-treated rats. Cumene hydroperoxide may have bound to the substrate binding site of the phenobarbital-inducible cytochrome P-450 form and inhibited the binding of aldrin. The constitutive forms of P-450 possibly were not affected by cumene hydroperoxide binding and this resulted in similar rates of metabolism of aldrin in control and phenobarbital-treated rats. The second explanation is more likely the reality because phenobarbital induces a specific cytochrome P-450 form (P-450IIB1) in the rat which is highly specific for aldrin.

Cumene hydroperoxide did not change the effect of β -naphthoflavone treatment on rat N-demethylation of aminopyrine. No difference between the cumene hydroperoxide and the NADPH-dependent reactions were noted. This finding was not unexpected since β -naphthoflavone is not known to induce the NADPH-cytochrome P-450 reductase in the rat. However, the decrease in the NADPH-dependent aldrin epoxidase activity observed in β -naphthoflavone-treated rats, was not present in the cumene hydroperoxide-dependent reaction. This contradicts the hypothesis that the decrease in the NADPH-dependent aldrin epoxidation activity with β -naphthoflavone was related to the induction of an isozyme with lower affinity

for aldrin. This may instead indicate that β -naphthoflavone treatment in the rat changes the vectorial alignment between the cytochrome P-450 forms and the reductase such that effective electron transfer is not achieved. When electrons were directly given the β -naphthoflavone induced cytochromes, a similar rate of metabolism was observed as for the controls.

As previously discussed, cumene hydroperoxide can interact with the binding domain of cytochrome P-450. This appears to be a problem for the phenobarbital-inducible P-450 to interact with aldrin but not with aminopyrine. This problem can be avoided by replacing cumene hydroperoxide with t-butyl hydroperoxide in the cytochrome P-450-mediated activities (Rahimtula and O'Brien, 1975; O'Brien and Rahimtula, 1980). The chemical structures of the two organic hydroperoxides (Figure 8) are markedly different. They differ in intrinsic electrophilicity and in steric requirements resulting in variation in regioselectivity (White and Coon, 1980).

Some differences between rat and chicken aminopyrine N-demethylase and aldrin epoxidase activities were observed with t-butyl hydroperoxide replacement of cumene hydroperoxide. In the chicken, the t-butyl dependent aminopyrine N-demethylation was unchanged for Sesbania drummondii, phenobarbital, and β -naphthoflavone treatments as compared to controls. The most likely explanation for this finding was that t-butyl

hydroperoxide is not very effective at contributing electrons and/or oxygen to the cytochrome P-450 forms that metabolize aminopyrine in the chicken. This was suggested by the very small rates of formaldehyde production observed in all groups.

As was observed in the chicken, t-butyl hydroperoxide is a poor mediator of aminopyrine N-demethylation in the rat. t-Butyl hydroperoxide-dependent aminopyrine N-demethylase specific activities were much lower than those for cumene hydroperoxide. However the differences among the treatment groups were the same with the two organic hydroperoxides. These differences were not evident when the activity was expressed per nmole of P-450 which also suggests t-butyl hydroperoxide is a poor and non-selective mediator of aldrin epoxidation.

t-Butyl hydroperoxide was also less effective in mediating aldrin epoxidation than was cumene hydroperoxide in the chicken. However, the differences among the treatment groups were still evident. Aldrin epoxidation appears to be a much more sensitive assay than aminopyrine N-demethylation and provides for a useful assay to distinguish the effects of t-butyl hydroperoxide with cumene hydroperoxide.

The utility of t-butyl hydroperoxide in studying organic

hydroperoxide-mediated metabolism was most evident for aldrin epoxidation in the rat. Cumene hydroperoxide was hypothesized to interfere with aldrin epoxidation in phenobarbital-treated rats resulting in equivalent rates of aldrin metabolism in both controls and phenobarbital-treated rats. This was confirmed with t-butyl hydroperoxide. An increase in aldrin epoxidase specific activity in phenobarbital-treated rats was observed with t-butyl hydroperoxide as has been previously observed in the NADPH-dependent reaction. However, the t-butyl hydroperoxide-dependent aldrin epoxidase molar activity for rats treated with phenobarbital was not different from controls which may suggest that phenobarbital induction of the reductase does contribute to some of the increase in activity. The explanation of the change in NADPH-dependent aldrin epoxidase activity by β -naphthoflavone treatment in rats received additional support with t-butyl hydroperoxide. The t-butyl hydroperoxide-dependent results agreed with the cumene hydroperoxide-dependent results in that control and β -naphthoflavone treated rats metabolize aldrin at similar rates and that the decreased activity observed in the NADPH-dependent reaction may be due to faulty electron transfer between the reductase and cytochrome components of the cytochrome P-450 monooxygenase system.

With the one exception of t-butyl hydroperoxide-dependent metabolism of aminopyrine in the chicken, no major differences

between cumene hydroperoxide and t-butyl hydroperoxide were observed in Sesbania drummondii-treated chickens or rats.

D. PHASE IV - INDUCTION OF MICROSOMAL ENZYMES BY SESBANIA DRUMMONDII AS DETERMINED BY SDS-PAGE AND WESTERN BLOTS WITH ANTI-CYTOCHROME P-450 ANTIBODIES. COMPARISON WITH EFFECTS CAUSED BY PHENOBARBITAL AND β -NAPHTHOFLAVONE.

OBJECTIVES:

1. To compare the effects of Sesbania drummondii, phenobarbital, and β -naphthoflavone on the SDS-PAGE patterns of chicken and rat hepatic microsomes.
2. To determine cross-reactivities of anti-cytochromes P-450 with proteins induced by Sesbania drummondii, phenobarbital, and β -naphthoflavone in chicken and rat hepatic microsomes.

MATERIALS AND METHODS:

A. Chemicals:

1. Sodium dodecylsulfate polyacrylamide gel electrophoresis (SDS PAGE): Acrylamide, N,N'-methylene-bis-acrylamide (bis), ammonium persulfate, N,N,N',N'-tetramethylethylenediamine (TEMED), and bromophenol blue were purchased from Bio-Rad Laboratories (Richmond, CA). Protein standards consisting of rabbit muscle phosphorylase B (97,400 MW), bovine serum albumin (66,000 MW), hen egg white ovalbumin (45,000 MW), bovine carbonic anhydrase (31,000 MW), soybean

trypsin inhibitor (21,500 MW), and hen egg white lysozyme (14,400 MW) were also obtained from Bio-Rad. Tris [hydroxymethyl] aminomethane hydrochloride (Tris-HCL buffer), glycine, and 2-mercaptoethanol were purchased from Sigma Chemical Co. (St. Louis, MO). Sodium dodecyl sulfate (SDS) was obtained from Mallinckrodt, Inc. (Paris, KY), and Coomassie blue R-250 was purchased from Amresco (Solon, OH).

2. Western blotting: Goat polyclonal antibodies raised against rabbit cytochrome P-450 form and LM2 (IIB1) were purchased from Oxford Biomedical Research, Inc. (Oxford, MI). Rabbit polyclonal antibodies to rat cytochrome P-450 forms b and c were generously provided by Dr. M.J.J. Ronis (Department of Pediatrics, University of Arkansas Medical School, Little Rock, AR). Goat anti-rabbit IgG-alkaline phosphatase conjugate and 5-bromo-4-chloro-3-indole phosphate (BCIP) were purchased from Bio-Rad Laboratories (Richmond, CA). Rabbit anti-goat IgG-alkaline phosphatase conjugate, p-nitro blue tetrazolium (NBT), tris[hydroxymethyl]aminomethane (Tris buffer), glycine, and Triton X-100 were obtained from Sigma Chemical Co. (St. Louis, MO). Sodium dodecylsulfate and methanol were obtained from Mallinckrodt, Inc. (Paris, KY). Nitrocellulose membranes were purchased from Schleicher and Schuell (Keene, NH), and 3MM chromatography paper was obtained from Whatman Limited (England). Dried milk powder (Carnation brand) and gelatin (Knox brand) were purchased from a local

grocery store. All other chemicals were reagent grade.

B. Animals:

Male White Leghorn chickens and Sprague-Dawley rats were obtained, cared for, and assigned to treatment groups as described in detail in Phase II.

C. Treatment:

Animals were treated with either bicarbonate-azide buffer (controls), *S. drummondii* extract, phenobarbital, or β -naphthoflavone as described in detail in Phase II.

D. Preparation of microsomes:

Microsomes were prepared from pooled livers of the subgroup, treated or control, as described in detail in Experiment 1, Phase I.

E. Protein determination:

The concentration of protein of the microsomal suspensions was determined as described in detail in Experiment 1, Phase I.

F. Sodium dodecylsulfate polyacrylamide gel electrophoresis
(SDS PAGE):

Discontinuous SDS-PAGE gels were prepared and run essentially according to the method of Laemmli (1970).

1. Preparation of gels: Slab gels were prepared at 10% (w/v) total monomer concentration from a stock solution containing acrylamide and bis at a ratio of 29.2 : 0.8. Tris-HCL buffer (pH 8.7) and SDS were included at final concentrations of 0.375 M and 0.1% (w/v), respectively. Monomer solutions were deaerated and polymerization was effected by addition of 0.05% each TEMED (v/v) and ammonium persulfate (w/v). Separating gels (ca. 80 x 60 x 0.75 mm) were cast and electrophoresed in a Bio-Rad Mini-Protein II apparatus. Stacking gels, comprised of 4% total monomer, 0.125 M Tris-HCL buffer (pH 6.9) and 0.1% SDS (w/v) were polymerized after deaeration via addition of 0.1% (v/v) TEMED and 0.05% (w/v) ammonium persulfate and were cast over separating gels with a teflon comb in place to form sample wells.

2. Preparation of samples: Protein samples were diluted to give a final concentration of 2 μ g/ml protein in a buffer containing 0.1 M Tris-HCL (pH 6.9), 16% (v/v) glycerol, 1.6% (w/v) SDS, 4% (v/v) 2-mercaptoethanol, and 0.008% (w/v) bromophenol blue, and boiled for 10 min. Aliquots of each

sample containing 30 μ g protein were underlaid in the sample wells after assembly of the "upper" electrode buffer chamber. A mixture of protein standards prepared in the same manner were included in one sample well.

3. Electrophoresis: The gel sandwiches and upper buffer chamber were assembled together with the lower buffer chamber, and electrode buffer containing 25 mM Tris (pH 8.3), 0.192 M glycine, and 0.1% (w/v) SDS was added. A potential difference of ca. 200 V was applied across the gel (ca. 30 mA/gel) with an EC105 Minicell (E-C Apparatus Corp., St. Petersburg, FL) constant voltage power supply for ca. 60 min until the tracking dye reached the bottom of the gel.

4. Staining and destaining: At the end of the run, electrophoresis was stopped and gels were removed from sandwiches. Gels were shaken in a staining solution containing 0.1% (w/v) Coomassie blue R-250, 40% (v/v) methanol, and 10% (v/v) acetic acid for at least 2 hr.

5. Measurement: Molecular weights were determined by comparing the relative mobility of the microsomal protein bands to the logarithms of the protein molecular weight markers.

6. Storage: Completed gels were dried between two layers of BioGelWrap (BioDesign Inc., Carmel, NY). Gels preserved in this way can be stored indefinitely.

G. Western blotting:

Electrophoretic transfer of proteins from SDS-polyacrylamide gels to nitrocellulose membranes was accomplished essentially by the method of Towbin et al. (1979). Immunoreactive proteins were detected with a secondary antibody-alkaline phosphatase conjugate used in conjunction with 5-bromo-4-chloro-3-indole phosphate and p-nitro blue tetrazolium (Blake et al., 1984).

1. Electrophoretic transfer: Transfer buffer, containing 0.25M Tris buffer (pH 8.3), 0.192 M glycine, 0.1% (w/v) SDS, and 20% (v/v) methanol, was prepared and prechilled to 4 C. Microsomal proteins were separated on SDS polyacrylamide gels. Gels were equilibrated in transfer buffer for at least 15 min. Nitrocellulose membranes were cut to the dimensions of each gel and soaked in transfer buffer for 15 to 30 min. Proteins were transferred from gels to nitrocellulose membranes in a Bio-Rad Trans-Blot electrophoretic transfer cell assembled according to the manufacturers directions. Briefly, a fiber pad saturated in transfer buffer was placed on the cathode-side panel of the gel holder and topped with a piece of chromatography paper cut

to fit the fiber pad. The pre-equilibrated gel was then laid atop the paper, the pre-wet membrane was placed on top of the gel, and a second piece of saturated chromatography paper covered the membrane. Care was taken to prevent the formation of air pockets between each layer. The blotting sandwich was completed by placing a second saturated fiber pad on top and closing the gel holder. The holder was mounted in the cell, ca. 2.5 L transfer buffer was added, and the buffer was stirred throughout the transfer. A potential difference of ca. 60 V (ca. 200 mA) was applied across the cell with an EC105 Minicell (E-C Apparatus Corp., St. Petersburg, FL) constant voltage power supply and transfer was completed in ca. 5 hrs.

2. Development of the blot: Membranes were removed from the blotting cell and shaken for two hrs at 37 C in a solution of 50 mM Tris buffer (pH 7.4), 0.2 M NaCl, and 0.05% (v/v) Triton X-100 (Tris-buffered saline or TBS) containing 5% (w/v) milk powder. Membranes were then washed by shaking for 10 min in two changes of TBS. Incubation with primary antibodies (anti-P-450) at recommended dilutions in TBS containing 1% (w/v) gelatin was effected at 37 C with shaking for 1 hr. Membranes were again washed by shaking for 10 min in two changes of TBS and incubation with the secondary antibody-alkaline phosphatase (AP) conjugate at the recommended dilution in TBS containing 1% (w/v) gelatin was

effected at 37 C with shaking for 1 hr. Membranes were washed by shaking for 10 min in 2-4 changes of TBS. Alkaline phosphatase-linked proteins were detected by reaction with NBT and BCIP: membranes were shaken in 25 mL of a solution of sodium bicarbonate (pH 9.8) and 2 mM MgCl_2 , containing 125 μl of 30 mg/mL NBT in 70% aqueous dimethylformamide and 125 μL of 15 mg/mL BCIP in dimethylformamide until purple bands appeared.

RESULTS:**1. SDS-polyacrylamide gel electrophoresis:**

Microsomal protein profiles from control, S. drummondii-, phenobarbital-, and β -naphthoflavone-treated chickens and rats are shown in Figures 9 and 10. The electrophoretic patterns of chicken microsomal proteins were similar for control, S. drummondii, phenobarbital, and β -naphthoflavone treatments but showed increased staining intensity of specific bands with the treatments. β -Naphthoflavone treatment produced the most distinct protein profile of the treated chickens with a markedly increased staining intensity of protein bands corresponding to molecular weights of 50,000, 53,500 and 56,000. Microsomes from phenobarbital-treated chickens produced two protein bands slightly more intense than controls corresponding to molecular weights of 53,500 and 56,000. S. drummondii treatment of chickens produced a dose-dependent increase in the staining activity of three protein bands corresponding to 50,000, 52,500, and 55,000 molecular weights.

The electrophoretic patterns of rat microsomes were similar for control and Sesbania drummondii treatments but were distinctly different for phenobarbital and β -naphthoflavone treatments. β -Naphthoflavone treatment markedly induced two protein bands of 52,500 and 55,000 molecular weights. Two microsomal protein bands of 55,000 and 60,000 molecular weights were also induced with phenobarbital-treated rat

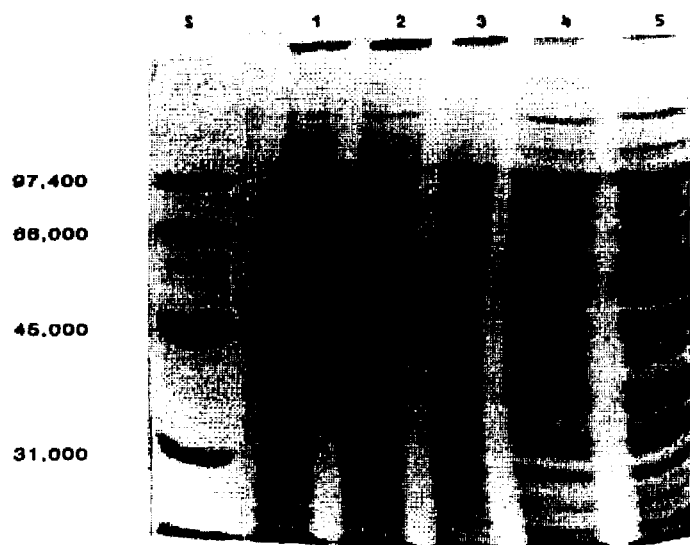


Figure 9. SDS-polyacrylamide gel electrophoresis of control and *Sesbania drummondii*, phenobarbital, and β -naphthoflavone-stimulated microsomal proteins of chickens.

Microsomal suspensions containing 30 μ g of proteins were loaded on a 10% separating gel and subjected to gel electrophoresis as described in the methods section.

Lane S: Molecular weight standards; Lane 1: Control chicken microsome; Lane 2: *Sesbania drummondii*-treated (0.5%) chicken microsome; Lane 3: *Sesbania drummondii*-treated (0.75%) chicken microsome; Lane 4: Phenobarbital-induced chicken microsome; Lane 5: β -naphthoflavone-induced chicken microsome.

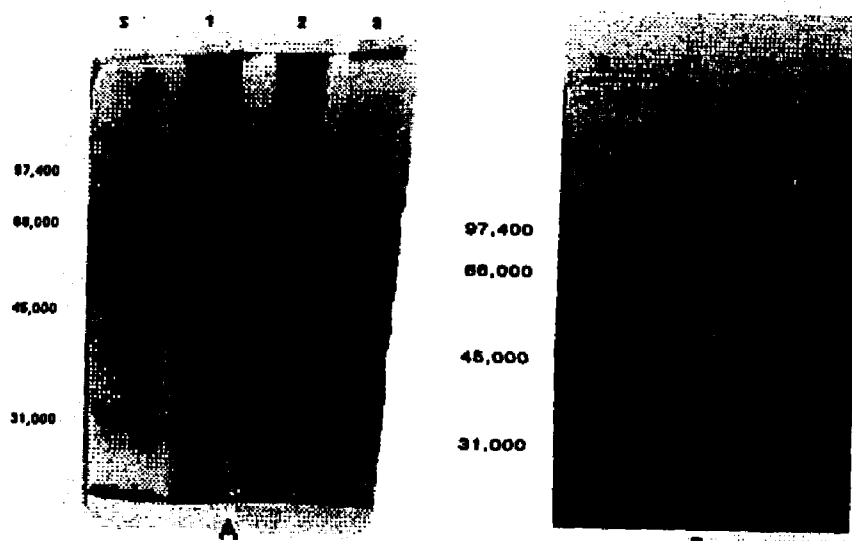


Figure 10. SDS-polyacrylamide gel electrophoresis of control and Sesbania drummondii, phenobarbital, and 8-naphthoflavone-stimulated microsomal proteins of rats.

Microsomal suspensions containing 30 μ g of proteins were loaded on a 10% separating gel and subjected to gel electrophoresis as described in the methods section.

- A. SDS-page of "classical" cytochrome P-450 inducers.
Lane S: Molecular weight standards; Lane 1: Control rat microsome; Lane 2: Phenobarbital-induced rat microsome; Lane 3: 8-naphthoflavone-induced rat microsome.
- B. SDS-page of Sesbania drummondii-treated microsome.
Lane S: Molecular weight standards; Lane 1: Control rat microsome; Lane 2: Sesbania drummondii-treated rat microsome; Lane 3: Phenobarbital-treated rat microsome.

microsomes. The electrophoretic pattern with S. drummondii treatment of rats exhibited similar protein bands to control microsomes, but the bands were of lower intensity.

2. Western blots:

The results of the cross-reactivity studies of chicken and rat hepatic microsomes with antibodies to rat cytochromes P-450IA1 and P-450IIB1 are summarized in Table 29. Strong cross-reactivity with the antibody against rat P-450IA1 was seen with β -naphthoflavone-treated rats and β -naphthoflavone- and S. drummondii-treated chickens (Figure 11). There were two protein bands recognized with rat anti-P-450IA1 in the Sesbania drummondii-treated chicken. One of the bands corresponded to the chicken β -naphthoflavone protein band and the other band was of a lower molecular weight. The control chicken microsomal protein showed weak cross-reactivity with this antibody. No cross-reactivity of the S. drummondii-treated rat microsomal protein was observed with the antibody against rat P-450IA1.

The antibody against rat cytochrome P-450IIB1 strongly cross-reacted with a microsomal protein from the phenobarbital-treated rat and weakly cross-reacted with the control rat microsomal protein (data not shown). All of the chicken microsomal proteins weakly cross-reacted with this antibody.

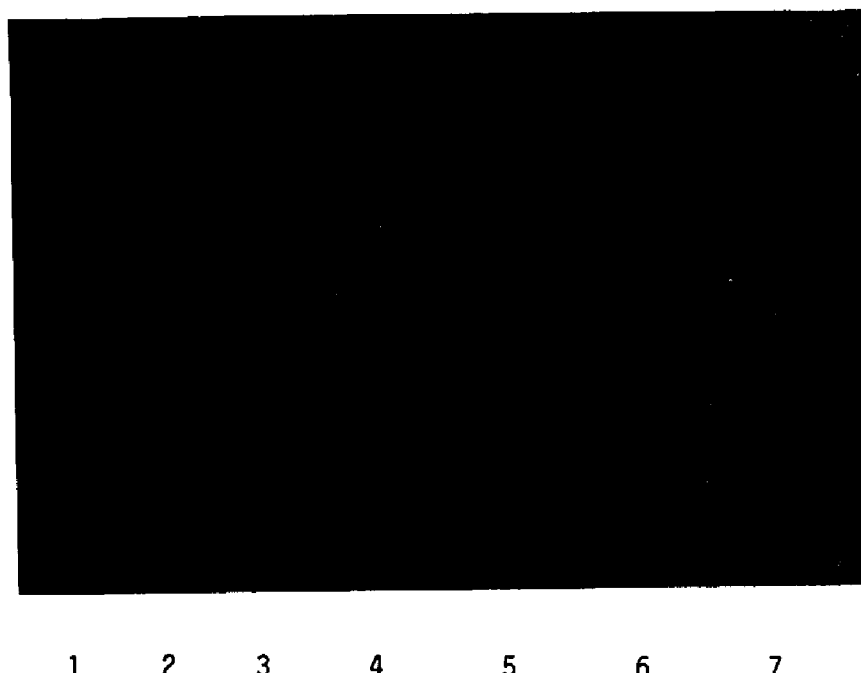


Figure 11. Western blot showing cross reactivity between antibodies to rat P-450 IA1 with liver microsome from rats induced by β -naphthoflavone and with liver microsomes from control, Sesbania drummondii-, and β -naphthoflavone-induced chickens.

Lane 1: Control rat microsome; Lane 2: β -naphthoflavone-induced rat microsome; Lane 3: S. drummondii-treated rat microsome; Lane 4: control chicken microsome; Lane 5: β -naphthoflavone-induced chicken microsome; Lane 6: S. drummondii- (0.5%) induced chicken microsome (0.5%); Lane 7: S. drummondii-induced chicken microsome (0.75%).

Table 29: Cross-reactivity detected in hepatic microsomes from control, *Sesbania drummondii*-, phenobarbital-, and -naphthoflavone-treated chickens and rats using polyclonal antibodies prepared against members of rat cytochrome P-450 isozymes.

Species	Treatment	Antibody to rat P-450	
		IA1	IIB1
Chicken	Control	+-	+-
	<i>S. drummondii</i> (0.5%)	2 bands	+-
	<i>S. drummondii</i> (0.75%)	2 bands	+-
	Phenobarbital	ND	+-
	β -Naphthoflavone	++	+-
Rat	Control	--	+-
	<i>S. drummondii</i>	--	--
	Phenobarbital	ND	++
	β -Naphthoflavone	++	--

++ Indicates cross-reactivity with a single protein band
 +- Indicates weak cross-reactivity with a single protein band
 -- Indicates no cross-reactivity
 ND Not determined

DISCUSSION:

SDS-gel electrophoresis has become a fundamental approach for the assessment of protein profiles of microsomal preparations. The discontinuous gel system of Laemmli (1970) has proven most effective for resolution of cytochromes P-450. Under optimal conditions isozymes differing in minimum molecular weights by only 500 daltons can be separated by this system (Ryan and Levin, 1990).

Induction in chicken and depression in rat hepatic microsomal proteins with Sesbania drummondii treatment were found using SDS-gel electrophoresis and confirmed the altered catalytic activities observed previously. This was indicated by increases in protein band staining intensity in the chicken microsomal proteins and decreases in protein band staining intensity in the rat microsomal proteins with S. drummondii treatment. The induction of cytochrome P-450 in the S. drummondii-treated chicken was also observed to be dose-dependent in gels.

The Sesbania drummondii-induced chicken microsomal protein profile as measured by the molecular weights of the protein bands did not closely parallel the protein profiles of phenobarbital or β -naphthoflavone. S. drummondii treatment resulted in induction of protein bands that were not induced by either the classical inducers. Few comparisons of the S.

drummondii gel protein profile to that of other inducers are possible because of the limited study of SDS-gel electrophoresis in avian microsomes. Hexachlorobenzene, reported to be a mixed-type of inducer in rats, was found in quail microsomal proteins to have an electrophoretic pattern similar to that produced by β -naphthoflavone (Carpenter et al., 1985). Methyl isobutyl ketone and n-hexane administered to adult White Leghorn hens increased protein bands of similar molecular weights to phenobarbital induction (Abou-donia et al., 1985). S,S,S-Tri-n-butyl phosphorothrithioate (DEF) was also shown in the White Leghorn hen to induce an electrophoretic protein profile similar to that of phenobarbital (Lapadula et al., 1984). Using electrophoretic analysis, Sundstrom et al. (1989) reported that 17 α -ethynylestradiol potentiated the two protein bands of 3-methylcholanthrene treatment in primary cultures of livers from 16- to 17-day old chicken embryos. Sinclair et al. (1989) observed that ethanol and glutethimide administered to cultured chick hepatocytes induced two microsomal proteins of 50,000 and 53,000 molecular weights. The proteins induced in the Sinclair et al. (1989) report were of a similar molecular weight to those induced in our Sesbania drummondii-treated chickens. However, caution in interpreting gel data is necessary because several cytochromes P-450 have identical minimum molecular weights, and other microsomal proteins such as epoxide hydrolase migrate to the same region of the gel (Lu

et al., 1975; Ryan and Levin, 1990). In addition, the value of the molecular weights for cytochromes P-450 as determined by the protein resolution may vary with electrophoretic conditions, including sources of reagents, buffer conditions, percentage of acrylamide, standards, and running time.

Sesbania drummondii treatment of rats resulted in a microsomal protein profile that was similar to that of control rat microsomal protein but with lower protein band staining intensity. There was no resemblance between the S. drummondii-treated rat microsomal protein profile and that of either phenobarbital or β -naphthoflavone.

Some of the above limitations of SDS-gel electrophoresis can be addressed through immunochemical identification. Immunoblotting techniques (western blots) have been used successfully to identify cytochromes P-450 in different tissues and species and to examine the influence of inducers on microsomal levels of certain isozymes (Ryan and Levin, 1990).

Cross-reactivity in western blots was used to examine the appearance of cytochrome P-450 isozymes resulting from Sesbania drummondii treatment that were structurally related to known, inducible rat forms. Antibody directed against rat cytochrome P-450IA1 detected two microsomal proteins from S.

drummondii-treated chickens, suggesting that treatment with an extract of Sesbania drummondii induces cytochrome P-450 forms in the chicken from the I gene family. One of the two protein bands detected corresponded with the protein band observed with β -naphthoflavone treatment which indicates that S. drummondii treatment induces an orthologue of rat P-450IA1 in the chicken. However, there was a second protein band detected in the S. drummondii-treated chicken microsomal protein that was not observed with any of the other treatments. There are several possible explanations for the detection of two protein bands with S. drummondii treatment of chickens. The first hypothesis is the second band may represent an orthologue to rat P-450IA2 (P-450d). This could be possible because the polyclonal antibody against P-450IA1 has been reported to cross-react with P-450IA2 isozyme of the rat (Ryan and Levin, 1990). Both cytochromes P-450IA1 and P-450IA2 are induced by 3-methylcholanthrene-type inducers in the rat. The presence of an orthologue in the chicken related to rat P-450IA2 has not, to the best of our knowledge, been reported. However, orthologues to this rat cytochrome have been detected in the mouse, rabbit, and human (Nebert et al., 1989b). This hypothesis may be discounted, however, because in our study β -naphthoflavone, which reportedly induces both P-450IA1 and P-450IA2 in the rat, induced only one protein band (supposedly P-450IA1) in both rats and chickens that cross reacted with the antibody against the rat P-450IA1. A

second hypothesis explaining the detection of the second band was that Sesbania drummondii treatment induced a new unique cytochrome P-450 isozyme in the chicken that shares an epitope to the rat P-450IA1 form. The third hypothesis is that the two protein bands observed with the S. drummondii-treated chicken microsomes were the result of protein degradation. However, this problem was unlikely since both bands were seen in the 50,000 to 60,000 molecular weight cytochrome P-450 region and microsomal protein samples kept for up to two years at 70 C have shown no change in pattern of cross-reactivity (Ronis et al., 1990).

The western blot results clearly indicated species differences in the effects of Sesbania drummondii treatment on cytochrome P-450 isozymes. In contrast to the two protein bands detected in chickens, no bands were detected in the rat S. drummondii-treated microsomal protein with the antibody against rat P-450IA1.

Less definitive conclusions about the western blot of Sesbania drummondii treatment of chickens and the antibody against rat P-450IIB1 can be drawn from our study because of the weak cross-reactivity of all the chicken groups with this antibody. Similar weak cross-reactivities to this antibody have been reported in a number of wild sea birds (Ronis et al., 1990). The lack of strong cross-reactivity of our phenobarbital-

treated chicken microsomal protein with the antibody against rat P-450IIB1 indicates that this rat antibody does not discriminate the phenobarbital-inducible cytochrome P-450 isozyme of the chicken. This finding, in addition to our reported difference in alkoxyphenoxazone metabolism, support the contention that phenobarbital induces different cytochrome P-450 forms in chickens versus rats.

The results of the western blot of the Sesbania drummondii-treated rat microsomal protein clearly indicate no detection by the antibody against rat P-450IIB1 suggesting that this treatment does not induce the cytochrome P-450IIB1 isozyme in the rat.

CHAPTER 5

SUMMARY AND CONCLUSIONS

Preliminary investigations indicated an extract of Sesbania drummondii induces different responses in chicken versus rat hepatic microsomal cytochrome P-450 monooxygenase systems. Groups of 4- to 8-week old White Leghorn chickens and Sprague-Dawley rats were given extracts of Sesbania drummondii by gavage for 3 days. Doses, which were 0.8% and 0.4% of daily body weights, respectively for chickens and rats, were adjusted to induce similar clinical changes in the two species. The hepatic microsomal cytochrome P-450 monooxygenase system of untreated control and treated animals were compared using cytochrome P-450 specific content, cytochrome b₅ specific content, NADH- and NADPH-cytochrome c reductase activity, and six cytochrome P-450-mediated activities. Increases of 2-fold in the cytochrome P-450 content, NADPH-cytochrome c reductase, aminopyrine N-demethylase, aniline hydroxylase, ethoxycoumarin O-deethylase, and aryl hydrocarbon hydroxylase activities; 4-fold in aldrin epoxidase activity; and 15-fold in ethoxyresorufin O-deethylase activity were observed in Sesbania drummondii-treated chickens. In contrast, the treated rats had nearly 2-fold decreases in these values. These findings indicated marked phylogenetic differences in control, regulation, or expression of the microsomal mixed function monooxygenase

systems between these avian and mammalian species in response to Sesbania drummondii extract.

The changes induced in the chicken and rat hepatic microsomal cytochrome P-450 monooxygenase system were not due to the sodium bicarbonate-sodium azide buffer used to prepare the Sesbania drummondii extract. The cytochrome P-450 specific content, aminopyrine N-demethylase and ethoxyresorufin O-deethylase activities were essentially the same in chickens and rats treated with the buffer versus animals not given the buffer. This was of particular concern because azide is an effective ligand and inhibitor of heme-iron containing enzymes such as catalase, peroxidase, and cytochrome oxidase (Keilin, 1936-1937; Smith et al., 1977; Winston and Cederbaum, 1982).

The loss of mixed function monooxygenase activity in the rat and the induced activity in the chicken probably were not due to a general loss of microsomal membrane stability or proliferation of endoplasmic reticulum, respectively as glucose-6-phosphatase, a microsomal membrane marker enzyme, was not changed by Sesbania drummondii treatment in either species. This was also confirmed by the fact that neither species exhibited changes in cytochrome b₅ specific content. This also indicates that there is no alteration in either species of general cytochrome anabolism or catabolism.

Additional studies focusing on the individual enzyme components as well as the functionality of the intact cytochrome P-450 monooxygenase system were conducted to further elucidate the effects of an extract of Sesbania drummondii on the chicken versus rat hepatic microsomal cytochrome P-450 system. The cytochrome P-450 enzyme component was assessed through characterization of cytochrome P-450 specific contents, cytochrome P-450 absorbance maxima, electrophoretic patterns, and immunochemical profiles of the hepatic microsomal proteins from chickens and rats treated with Sesbania drummondii. The NADPH-cytochrome P-450 reductase component was evaluated by measuring NADPH-cytochrome c reductase activities and by determining the relative contribution of this enzyme to the overall changes induced by Sesbania drummondii on various microsomal cytochrome P-450-dependent monooxygenase activities. This was accomplished by comparing organic hydroperoxide-mediated (NADPH-independent) metabolism with NADPH-dependent metabolism of two cytochrome P-450 substrates. All of these studies were also performed with phenobarbital and β -naphthoflavone treatments (two "classical" cytochrome P-450 inducers) of chickens and rats for comparison.

Sesbania drummondii treatment induced unique profiles in the catalytic properties of the chicken versus rat hepatic cytochrome P-450 monooxygenase system. Groups of White

Leghorn chickens and Sprague-Dawley rats were treated with either bicarbonate-azide buffer (control), Sesbania drummondii extract, phenobarbital, or β -naphthoflavone. The Sesbania drummondii-treated rats received daily doses equal to 0.5% of body weight and the chickens received 0.5% or 0.75% of body weight. The catalytic properties of the hepatic cytochrome P-450 monooxygenase system were determined by characterizing cytochrome P-450-mediated enzyme activities of both low and high degrees of specificity for cytochrome P-450 isozyme families. The activities studied were aminopyrine N-demethylase, aldrin epoxidase, and the O-dealkylation of various alkyl-substituted alkoxyphenoxazones. With few exceptions, Sesbania drummondii induced catalytic changes differently than those induced by the two "classical" inducers, phenobarbital and β -naphthoflavone.

Aminopyrine N-demethylase activity has been associated with the phenobarbital-inducible cytochrome P-450 isozyme family (P-450IIB1). With Sesbania drummondii treatment, the specific activity of aminopyrine N-demethylase was increased 2-fold in chickens and decreased 2-fold in rats. In comparison, phenobarbital treatment increased aminopyrine N-demethylase specific activity nearly 3-fold in both chickens and rats and β -naphthoflavone treatment increased the activity in the chicken by 2-fold but did not alter the activity in the rat. Aminopyrine N-demethylase activity did not effectively

discriminate between the different induced cytochrome P-450 isoforms as the induced rates of this activity with phenobarbital were small in both species and in the chicken there was little distinction between the rates with phenobarbital and β -naphthoflavone induction.

Aldrin epoxidase activity has also been associated with phenobarbital-inducible cytochrome P-450 but with greater specificity. Aldrin epoxidation in the Sesbania drummondii-treated chicken paralleled phenobarbital treatment, whereas in the treated rat this activity paralleled β -naphthoflavone treatment. The aldrin epoxidase specific activity was increased 4-fold in chickens and decreased 2-fold in rats with Sesbania drummondii treatment. In comparison, phenobarbital increased aldrin epoxidase specific activity by 5-fold in both chickens and rats. β -Naphthoflavone-treatment had no effect on chicken aldrin epoxidation and in the rat decreased this activity nearly 2-fold.

The O-dealkylation of various alkoxyphenoxazone derivatives are presently the most specific of the cytochrome P-450-mediated activities for distinguishing between the inducible cytochrome P-450 isozymes in rat hepatic microsomes (Burke and Mayer, 1983; Burke et al., 1985). Studies in birds, thus far, have been limited to the ethoxy-derivative of hydroxyphenoxazone. To the best of our knowledge, the utility

of the hepatic microsomal metabolism of various substituted alkoxyphenoxazones as probes for the phenobarbital and β -naphthoflavone induction of cytochrome P-450 in chickens has not been described.

The chicken exhibited markedly different induction patterns of the alkoxyphenoxazone O-dealkylases to phenobarbital and β -naphthoflavone than did the rat. As anticipated β -naphthoflavone treatment of rats resulted in a marked increase (18-fold) of ethoxyphenoxazone O-dealkylase activity with much smaller increases with the other substrates. In contrast, the highest increased activity in the β -naphthoflavone-treated chicken was benzyloxyphenoxazone O-dealkylation (32-fold) followed closely by ethoxyphenoxazone O-dealkylation (27-fold). In good agreement with the literature, phenobarbital treatment of the rat resulted in very large increases in pentoxy- (197-fold) and benzyloxyphenoxazone O-dealkylation (98-fold). However, none of the alkoxyphenoxazone O-dealkylase activities were induced in the phenobarbital-treated chicken. In fact, the O-dealkylation of pentoxy- and benzyloxyphenoxazone were actually decreased in the phenobarbital-treated chicken. These data suggest benzyloxy- and ethoxyphenoxazone are useful probes for distinguishing β -naphthoflavone inducible cytochrome P-450 isozymes in chickens. However, in marked contrast to the rat enzymatic system, none of the alkoxyphenoxazone O-dealkylase activities

appear to be induced in the phenobarbital-treated chicken.

The catalytic profile of the alkoxyphenoxazone O-dealkylases clearly distinguished Sesbania drummondii treatment from phenobarbital and β -naphthoflavone induction in the rat and β -naphthoflavone induction in the chicken. Sesbania drummondii treatment of rats induced marked decreases in pentoxy- and benzyloxy O-dealkylase specific activities, whereas, phenobarbital treatment induced marked increases in these same activities. Ethoxyphenoxazone O-dealkylase, the activity markedly increased with β -naphthoflavone treatment, was unchanged in the rat with Sesbania drummondii treatment.

Microsomal-dependent alkoxyphenoxazone metabolism in chickens administered Sesbania drummondii extract was clearly distinguishable from that of chickens given β -naphthoflavone. β -Naphthoflavone treatment markedly increased the O-dealkylation of ethoxy-, pentoxy-, and benzyloxyphenoxazone, whereas S. drummondii treatment caused no induction of these activities. This finding contradicted the preliminary finding that S. drummondii markedly induced ethoxyresorufin O-dealkylation. The reason for this discrepancy between our two studies is not known. The two studies were different in the year the S. drummondii crop was harvested, the methods of the assay, and in the laboratory and personnel where the assay was performed.

Phenobarbital and S. drummondii treatments in the chicken shared a similar pattern of metabolism of pentoxyphenoxazone suggesting induction of cytochrome P-450IIB1 by the extract. However this data must be interpreted with caution in the chicken because both treatments significantly decreased the pentoxyphenoxazone O-dealkylase specific activity in contrast to the marked increase in this activity observed with phenobarbital treatment in the rat.

In summary, the catalytic properties of hepatic microsomes from Sesbania drummondii-treated animals showed some similarities with phenobarbital treatment in the chicken but also differences with phenobarbital and β -naphthoflavone treatments in both species.

Sesbania drummondii induced mostly opposite effects on the total specific content of hepatic microsomal cytochrome P-450 in chickens versus rats. Total cytochrome P-450 specific content i.e., a measure of all the cytochrome P-450 forms, was increased 2-fold in chickens but decreased 2-fold in rats with S. drummondii treatment. The magnitude of the increase induced in treated chickens was similar to that of phenobarbital-induced chickens. The absorbance maximum (Soret maximum) of the carbon monoxide-reduced difference spectrum was the only measure of the cytochrome P-450 enzyme component that was similarly not affected in both the chicken and rat by

S. drummondii treatment. Neither species expressed shifts in their absorbance maximum away from the uninduced levels. This was a distinct difference from β -naphthoflavone treatment in which a characteristic blue shift in the peak absorption to 448 nm, occurred in both chickens and rats. Induction in the chicken and depression in the rat hepatic microsomal proteins with Sesbania drummondii treatment were confirmed using SDS-gel electrophoresis. This was evident by increased protein band staining intensity in the chicken microsomal proteins and decreased protein band staining intensity in the rat microsomal proteins. The protein profile as measured by the molecular weight of the protein bands appeared to be unique with S. drummondii treatment of chickens because it did not closely parallel phenobarbital- or β -naphthoflavone-treated chicken microsomal protein profiles. In contrast, the S. drummondii-treated rat microsomal protein profile was very similar to that of control rat microsomal protein but of lower protein band staining intensity. Again, there was no resemblance of the S. drummondii-treated rat with either phenobarbital or β -naphthoflavone. The strongest evidence of species differences in the response of the hepatic cytochrome P-450 monooxygenase system to Sesbania drummondii treatment was from immunochemical studies. Immunoblotting techniques (western blots) are best able to discriminate among the cytochrome P-450 forms. Sesbania drummondii treatment of chickens induced two microsomal proteins that were detected

with antibody against rat cytochrome P-450IA1, the major isozyme induced by 3-methylcholanthrene. One of these two proteins corresponded with the protein of β -naphthoflavone-treated chickens which was also detected with this antibody. This indicated that S. drummondii treatment induces an orthologue of rat cytochrome P-450IA1 in the chicken. However, the second protein band in the Sesbania drummondii-treated chicken, which was of a lower molecular weight, appeared to be unique to this treatment. This second microsomal protein may be a novel chicken cytochrome P-450 isozyme induced with S. drummondii treatment that shares an epitope to the rat cytochrome P-450IA1 form. However, these two Sesbania drummondii-induced cytochrome P-450 isoforms did not exhibit the characteristic substrate specificities of cytochrome P-450IA1 isozymes in chickens or rats. Most notable was the lack of induction of the O-dealkylation of ethoxyphenoxazone in the S. drummondii-treated chickens versus the marked inductions observed with β -naphthoflavone. Neither of these microsomal proteins seen in the chicken were detected by this antibody in the S. drummondii-treated rat. An antibody directed against the major phenobarbital-inducible rat P-450 isozyme, P-450IIB1, was shown to weakly cross-react with chicken S. drummondii-treated microsomes. However, this cross-reactivity appeared to be non-specific as both control and all treated chickens weakly cross-reacted with this antibody.

NADPH-cytochrome P-450 reductase was also differently affected by Sesbania drummondii-treatment in chickens versus rats. Sesbania drummondii treatment induced a 2-fold increase in NADPH-cytochrome c reductase activity in chickens and no change in this activity in rats. This suggested that the rate of electron transfer from NADPH-cytochrome P-450 reductase to the cytochrome P-450-substrate complex in the chicken might be altered as a result of S. drummondii treatment. In that regard, the reduction of cytochrome P-450-substrate complex is considered by many as the rate-limiting step in cytochrome P-450-dependent monooxygenase activity (White and Coon, 1980). Therefore, alterations of this enzyme, either quantitative or qualitative, may be envisaged to alter cytochrome P-450-dependent activity. The effect of induction of NADPH-cytochrome P-450 reductase by S. drummondii to the overall metabolic function of cytochrome P-450-dependent monooxygenase activity was evaluated by measuring the peroxygenase activity of cytochrome P-450 which proceeds independently of the contribution of the reductase by forming directly the divalently-reduced peroxy P-450-substrate complex (Kadlubar et al., 1973; Rahimluta and O'Brien, 1974). This was accomplished by comparing the cytochrome P-450-mediated metabolism of aminopyrine and aldrin catalyzed by the classical NADPH-dependent cytochrome P-450 reductase i.e., the oxygenase activity of cytochrome P-450 to that catalyzed by the organic hydroperoxide-dependent peroxygenase activity of

cytochrome P-450 (NADPH-independent system). This report is the first evaluation of the organic hydroperoxide-mediated metabolism of aminopyrine in the chicken and perhaps more importantly, of an organic hydroperoxide-mediated aldrin epoxidation in the chicken and the rat. This may have important mechanistic implications in that highly electrophilic epoxides are now shown to be generated via a peroxidaic mechanism from a chlorinated hydrocarbon insecticide.

Differences in the metabolism of aminopyrine were observed in hepatic microsomes from chickens treated with S. drummondii as a function of the NADPH- versus cumene hydroperoxide-dependent reactions. This suggests that in the chicken, S. drummondii-induced changes in the NADPH-cytochrome P-450 reductase component does contribute to the increased aminopyrine N-demethylase activity. In contrast, the metabolism of aldrin was affected similarly with both NADPH or cumene hydroperoxide components in the S. drummondii-treated chicken. This implied that induction of microsomal mixed-function oxidase activity by Sesbania drummondii includes induction of an isozyme or family of isozymes in the chicken that are involved in the metabolism of aldrin and that the induction of the reductase component was not critical to this metabolism.

In the Sesbania drummondii-treated rat, there were similar decreases in aldrin epoxidation and aminopyrine N-demethylation regardless of whether the NADPH- or cumene hydroperoxide-dependent reactions were employed. Thus it would not appear that the decreases in metabolism by rat liver microsomes were due to less effective electron transfer from the reductase. One possible explanation for this is that S. drummondii actually altered the cytochrome P-450 isozyme population such that it less effectively metabolizes these substrates. Indeed our observation of less cytochrome P-450 protein band staining intensity in S. drummondii-treated rats using SDS-PAGE supports this contention.

Comparison of NADPH- versus cumene hydroperoxide-dependent aldrin metabolism yielded unexpected results with the phenobarbital-treated rat. Although a marked increase in the NADPH-dependent epoxidation of aldrin was noted with the phenobarbital-treated rat (5-fold), such an increase was not evident with the cumene hydroperoxide-dependent system. Cumene hydroperoxide has been shown to bind the hydrophobic domain of cytochrome P-450 which in some cases effectively interferes with the binding of certain substrates (Rahimtula and O'Brien, 1975; O'Brien and Rahimtula, 1980). Therefore, additional studies utilizing t-butyl hydroperoxide, a smaller less bulky organic hydroperoxide, that unlike cumene hydroperoxide, does not interact strongly with the cytochrome

P-450 binding domain were undertaken. When t-butyl hydroperoxide was utilized with the phenobarbital-treated rat microsome, the increase in aldrin activity was again apparent suggesting that the apparent induction of aldrin epoxidase activity was not expressed with cumene hydroperoxide owing to non-specific interactions with the hydrophobic binding domain. No differences were detected between the two organic hydroperoxides on the S. drummondii-induced treatment effects on the metabolism of aminopyrine or aldrin in either chickens or rats.

In conclusion, it appears Sesbania drummondii treatment induces both the cytochrome P-450 and NADPH-cytochrome P-450 reductase enzyme components of the hepatic microsomal cytochrome P-450 system of chickens. In contrast, only the cytochrome P-450 component is depressed in the rat with S. drummondii treatment. A number of chemically diverse compounds have also been reported to inhibit cytochrome P-450-dependent activity in rats. These compounds include an essential element, manganese (Deimling and Schnell, 1984); a hepatotoxicant, carbon tetrachloride (Sesardic et al., 1989); as well as the therapeutically useful antifungal agents, ketaconazole, miconazole, and clotrimazole (Rodrigues et al., 1987) and the antibacterial agent, chloramphenical (Halpert et al., 1988).

Moreover, studies on the peroxygenase activity of cytochrome P-450 support the idea that increase in MFO activity following Sesbania drummondii treatment in chickens were due to alterations in the content and isozyme profiles of cytochrome P-450 and not due to alterations in electron-transfer efficiency. This was borne out by the immunological studies which indicated the presence of new immunoreactive proteins in the molecular weight range of cytochrome P-450.

The Sesbania drummondii extract in chickens appears to induce at least two cytochrome P-450 isozymes. An immunochemical study revealed that these two proteins cross-react with the antibody against rat cytochrome P-450IA1. One of these proteins may be a 3-methylcholanthrene-inducible P-450 form of the I cytochrome P-450 gene family. The other may be a novel cytochrome P-450 form induced by Sesbania drummondii. Interestingly, these two proteins did not show typical 3-methylcholanthrene-type catalytic activities. In fact, their catalytic activities were more like that of phenobarbital. Only specific isolation and gene sequencing will permit definitive identification and classification of the S. drummondii-induced chicken cytochrome P-450 isozymes.

In contrast to the chicken, the rat hepatic cytochrome P-450 monooxygenase system was decreased with the Sesbania drummondii extract. This appears to be the result of a

general depression of the constitutive cytochrome P-450 isozymes rather than effects on specific cytochrome P-450 isozymes as gel electrophoresis indicated that all proteins that had molecular weights similar to the cytochrome P-450's were less intense. Neither NADPH-cytochrome P-450 reductase nor the integrity of the endoplasmic reticulum appears to be altered in the rat. Two likely mechanisms for the S. drummondii-induced effect in the rat are 1) Sesbania drummondii depresses the synthesis of new cytochrome P-450 protein, i.e., down-regulation; or 2) Sesbania drummondii enhances the post-transcriptional and/or post-translational catabolism of cytochrome P-450 in a manner reminiscent of the ethanol inducible cytochrome P-450 discussed in the introduction to this thesis.

The Sesbania drummondii treatment used in this study was a crude plant extract which is composed of a number of different chemical compounds. Undoubtedly a number of the different chemical components contained in the extract are involved in the differences observed in the chicken versus rat hepatic microsomal cytochrome P-450 systems. This is not surprising owing to the extreme pharmacological response differences to a variety of chemical agents contained in plants. Another plant that induces different toxicosis depending on the animal species is Bracken fern (Pteridium aquilinum) (Cheeke and Shull, 1985). Nonruminants (horses, pigs) may develop a

thiamine deficiency, while cattle and sheep may exhibit signs of a fatal hemorrhagic syndrome. Bracken also contains a carcinogen which may affect all species, including humans. Natural toxicants in poisonous plants have had significant effects on livestock production in North America. Many of these natural toxicoses, such as fescue foot, summer fescue toxicosis, bracken poisoning, and vetch poisoning of cattle, still have not conclusively had their toxic agents defined (Cheeke and Shull, 1985). However, their pathophysiological effects have been vigorously pursued even in the absence of the knowledge of the specific toxic agent using crude extracts of these plants. Many of these studies have resulted in the discovery of useful preventive and treatment measures. Therefore, regardless of the specific chemical(s) in the extract and the specific isozyme(s) of cytochrome P-450 affected, it is clear that Sesbania drummondii can alter the hepatic microsomal cytochrome P-450 monooxygenase system of mammals and birds with important expected consequences on xenobiotic and endobiotic metabolism.

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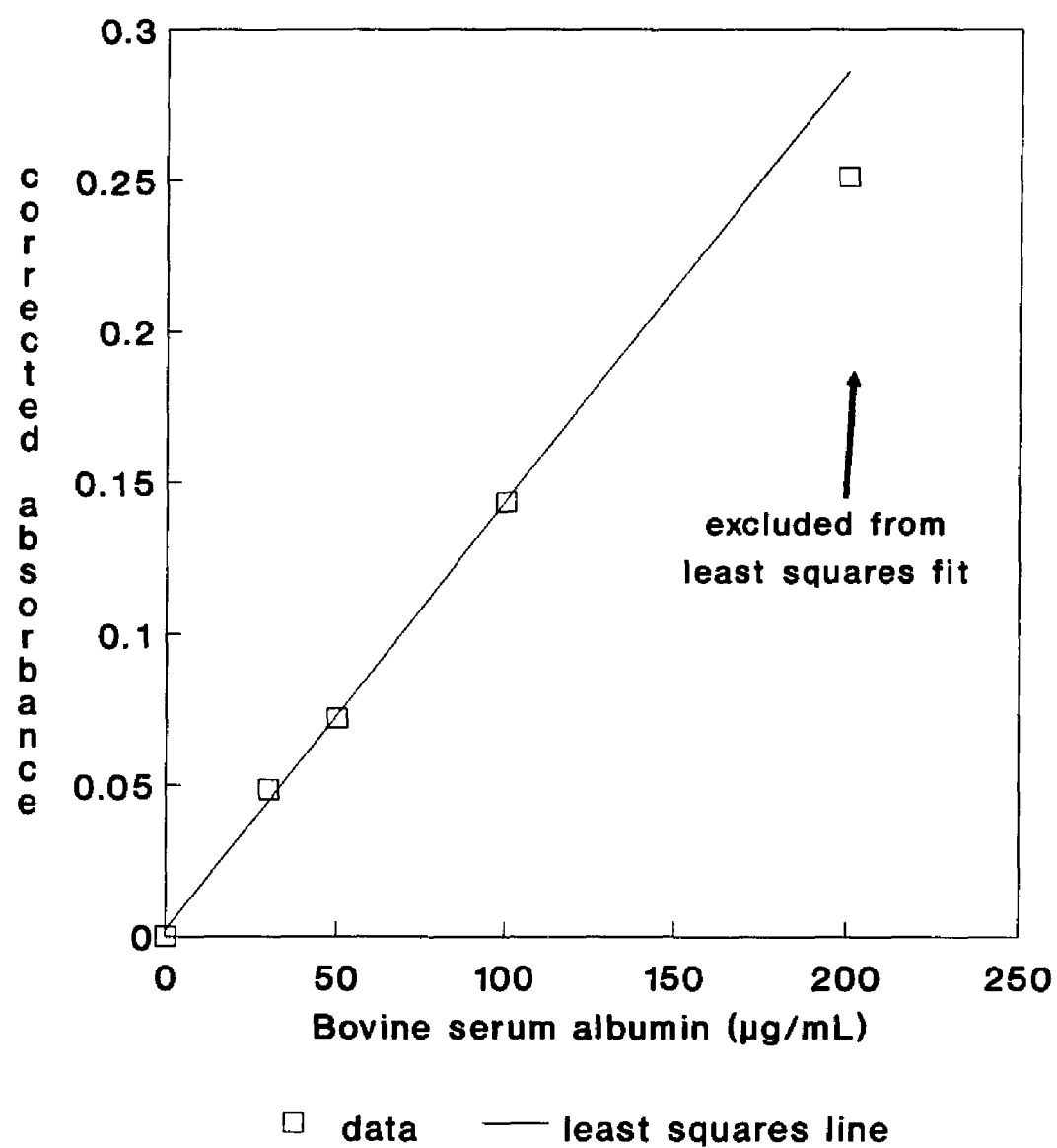
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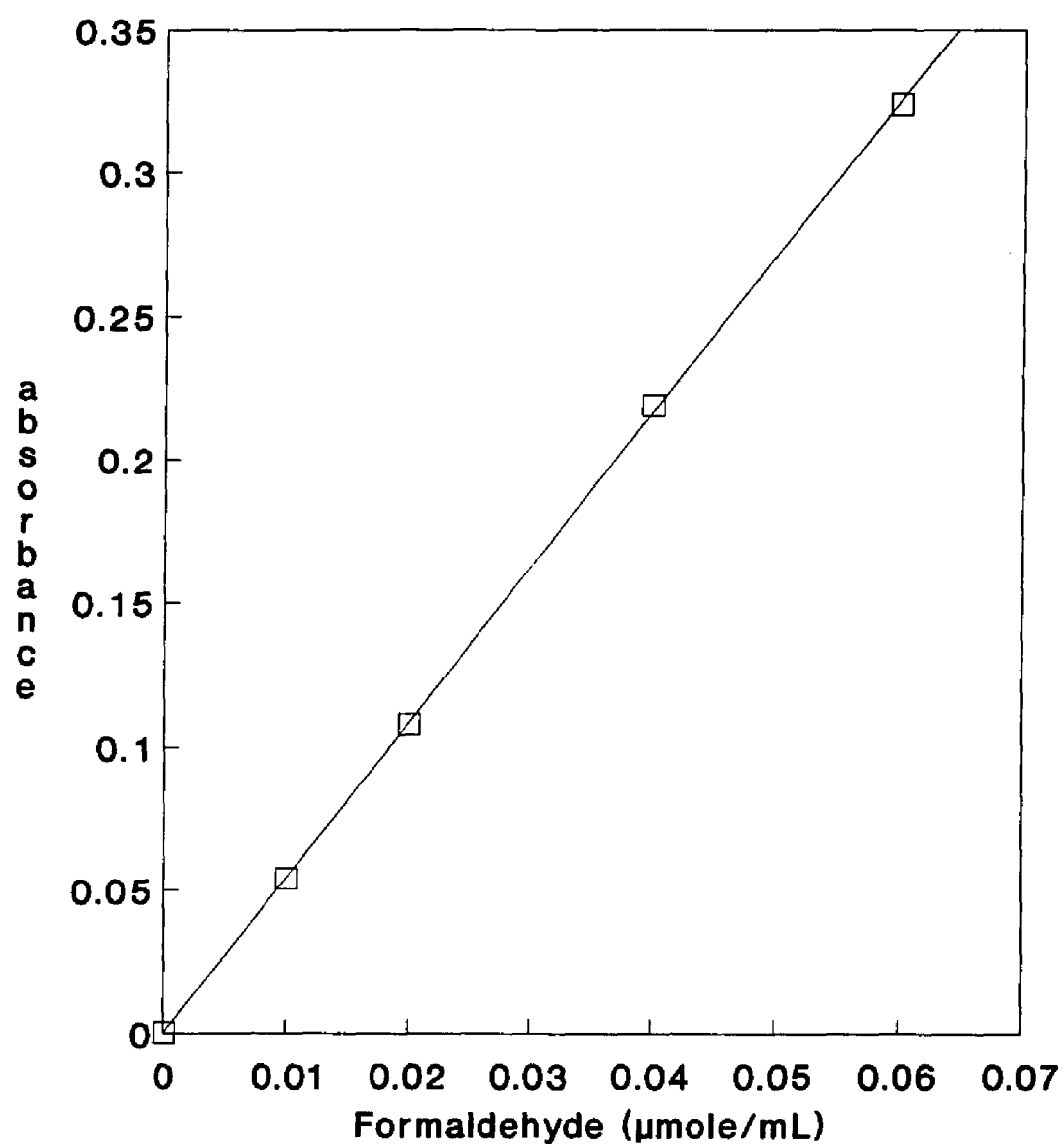
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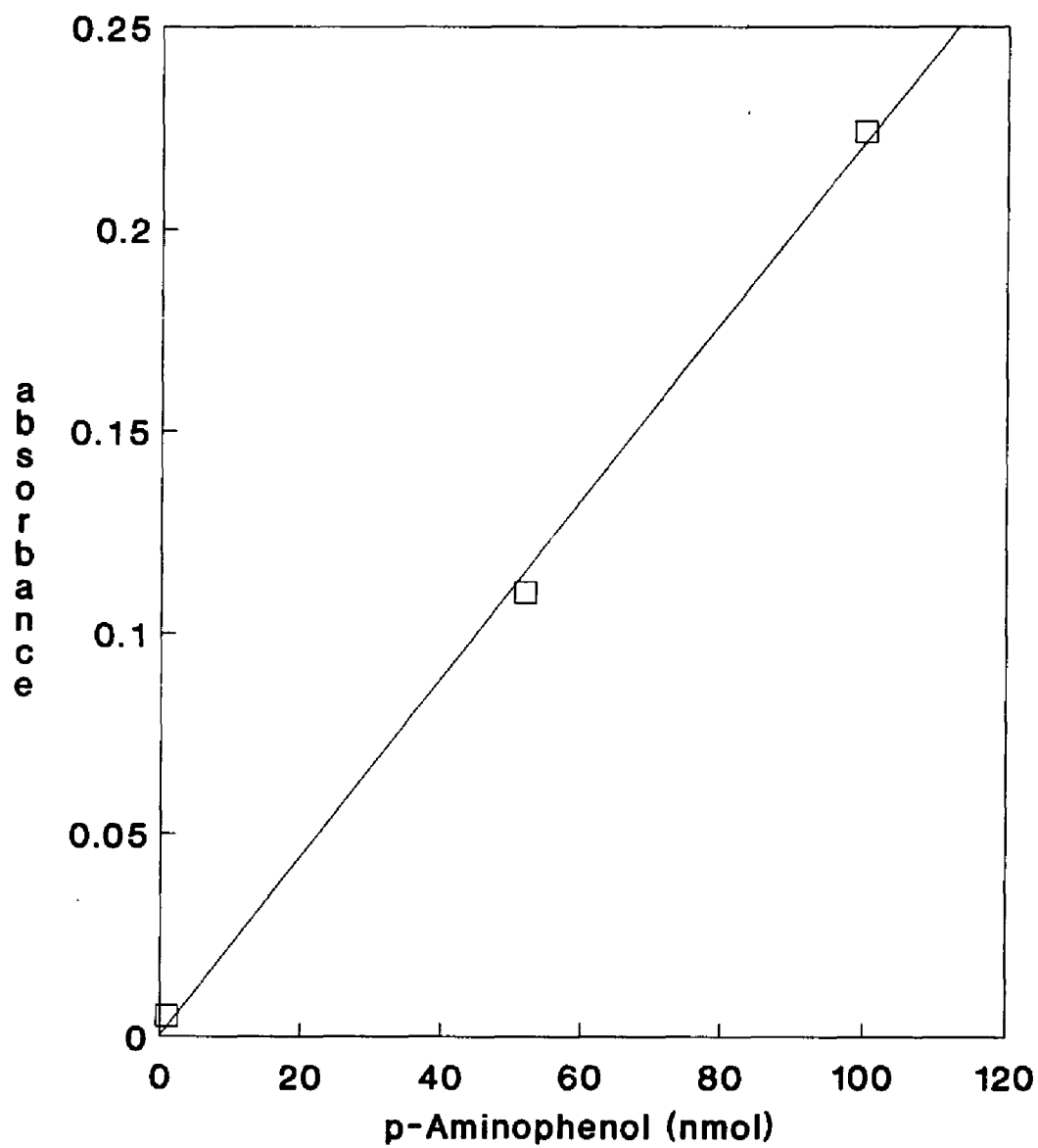


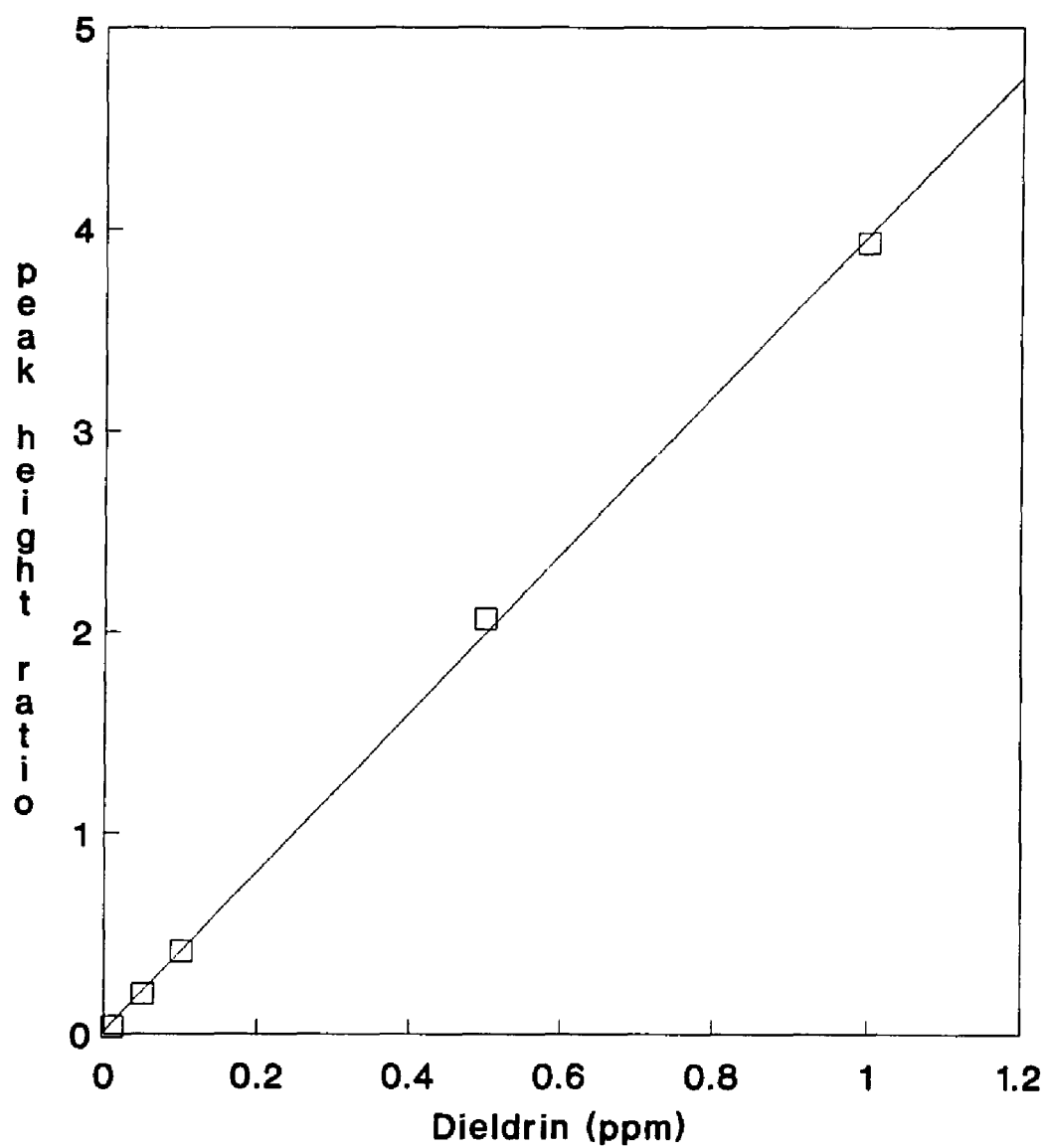
Appendix 1. Lowry protein standard curve



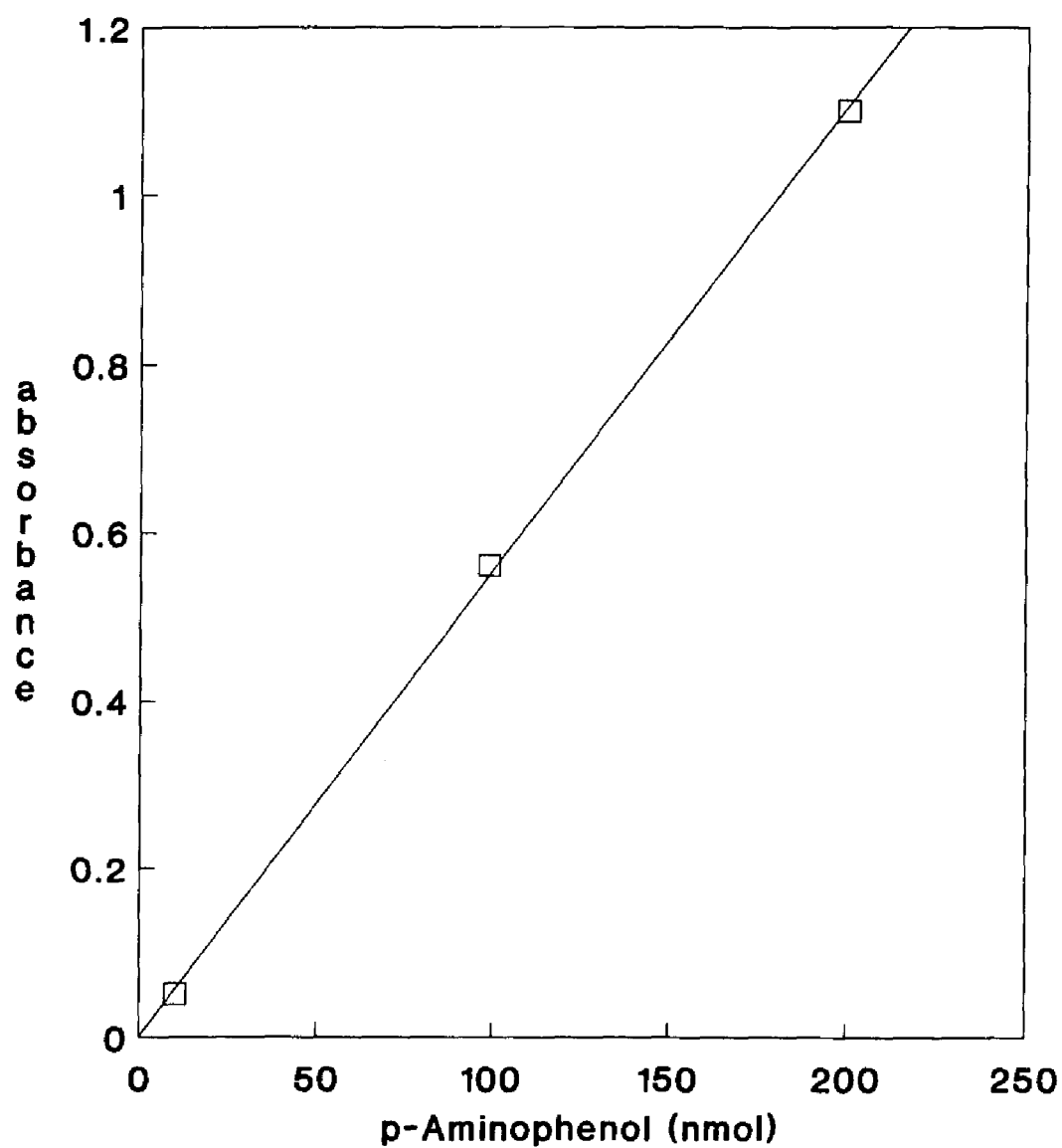
**Appendix 2. Formaldehyde standard
curve for the aminopyrine
N-demethylase assay**

**Appendix 3. p-Aminophenol standard
curve for the aniline hydroxylase
assay**

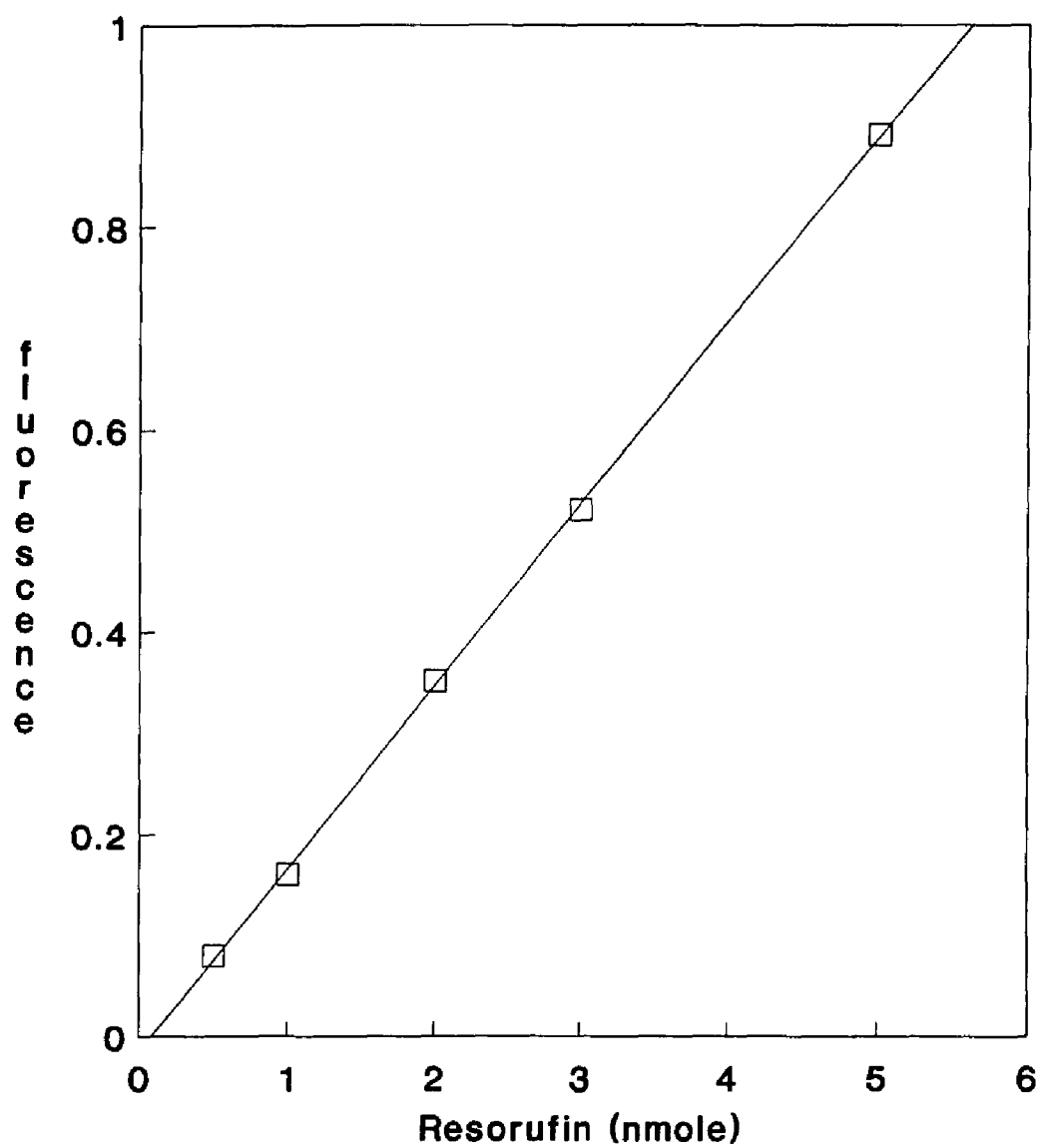




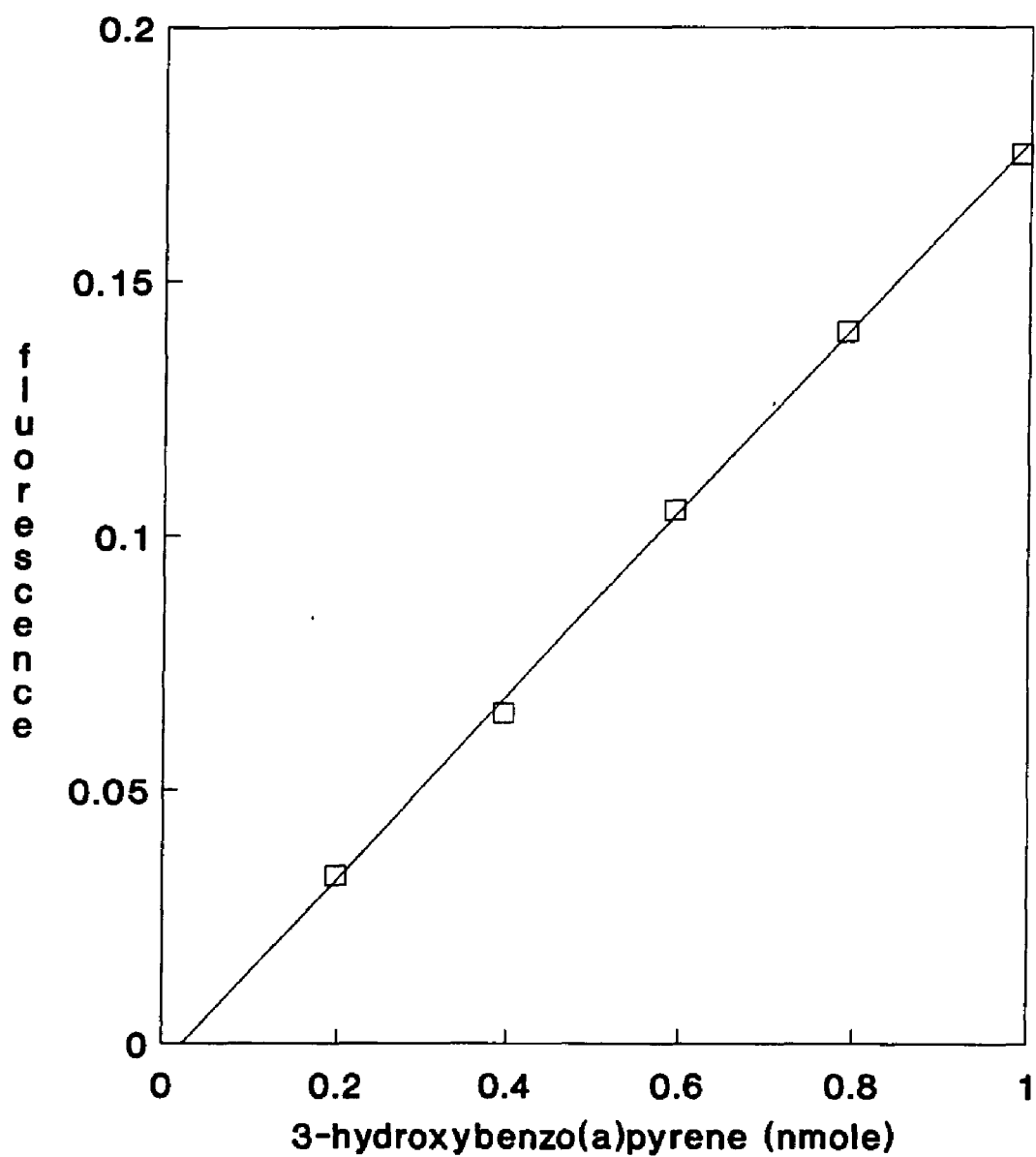
**Appendix 4. Dieldrin standard
curve for the aldrin epoxidase
assay**



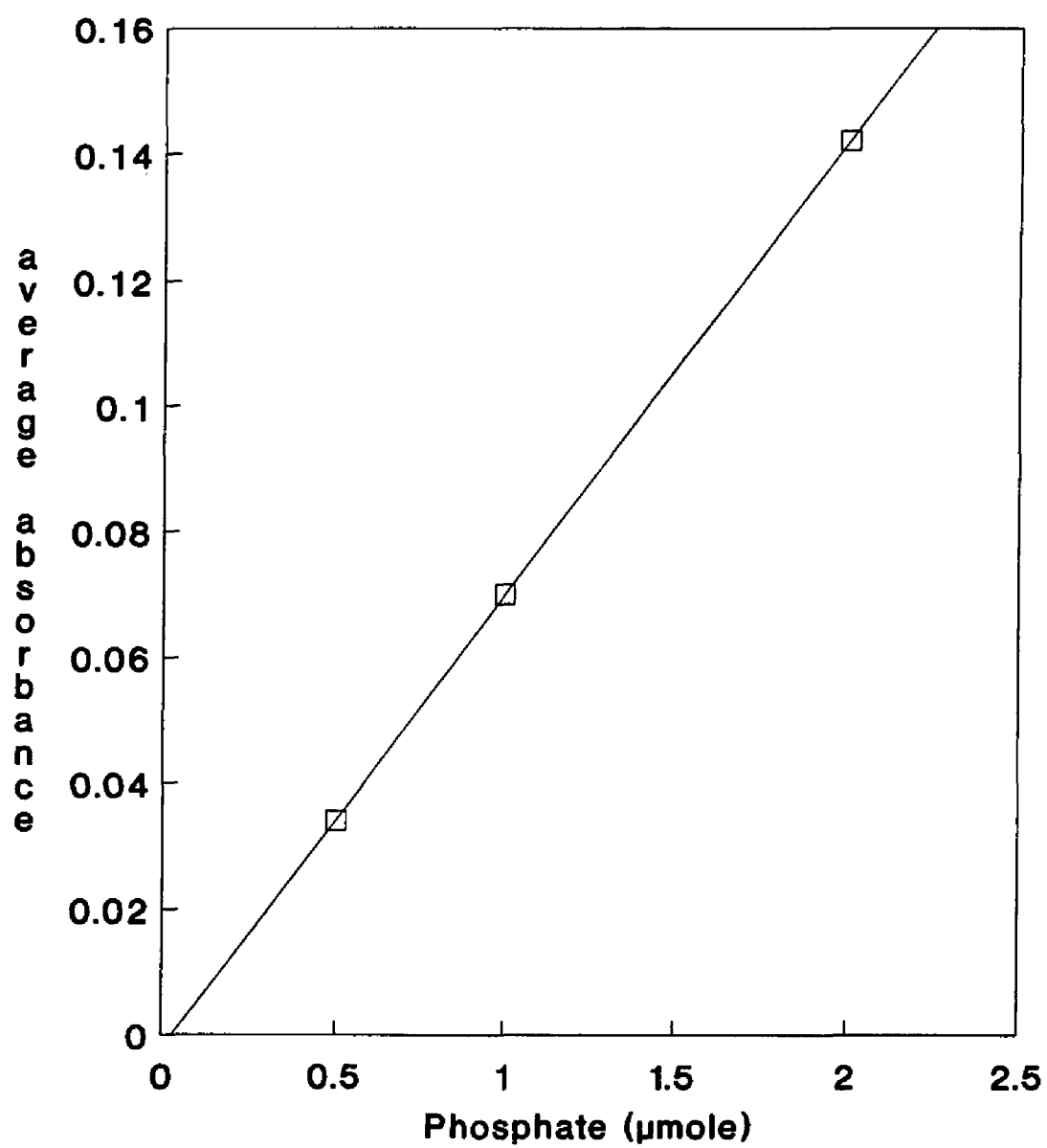
Appendix 5. 7-Hydroxycoumarin standard curve for the ethoxycoumarin O-deethylase assay.



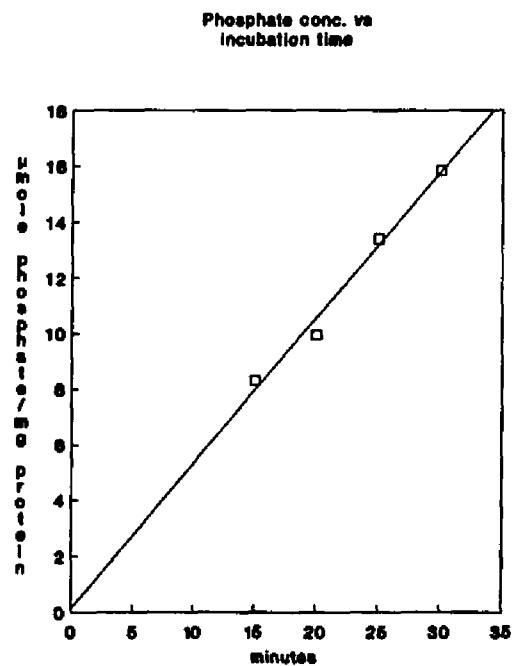
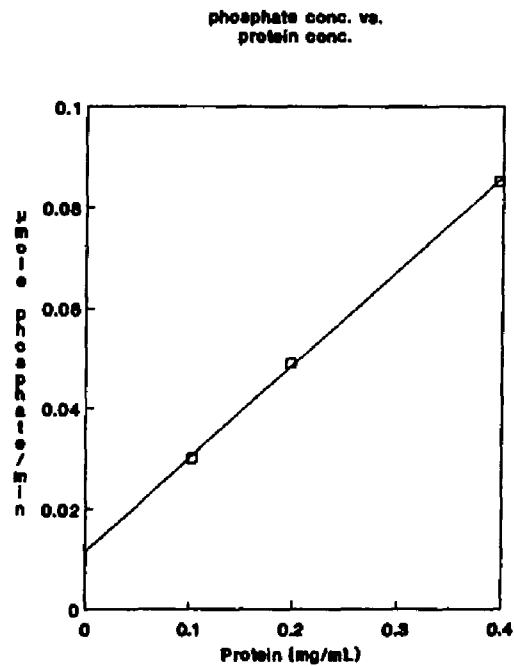
**Appendix 6. Resorufin standard curve
for the ethoxyresorufin
O-deethylase assay.**



**Appendix 7. 3-Hydroxybenzo(a)pyrene
standard curve for the aryl hydro-
carbon hydroxylase assay.**

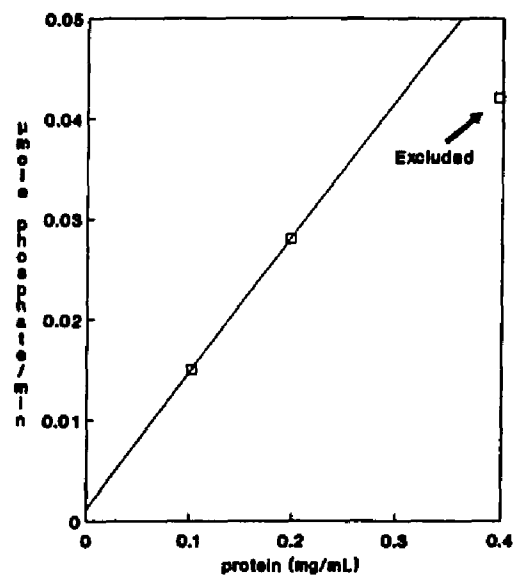


**Appendix 8. Phosphate standard
curve for the
glucose-6-phosphotase assay.**

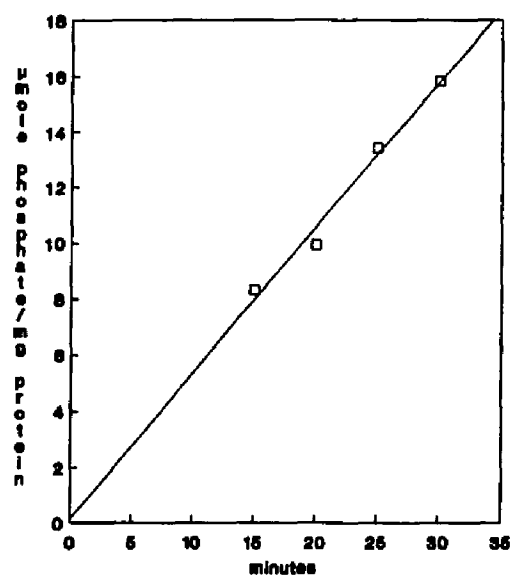


Appendix 9. Phosphate concentration vs. protein concentration and incubation time in the chicken hepatic microsomal glucose-6-phosphatase assay.

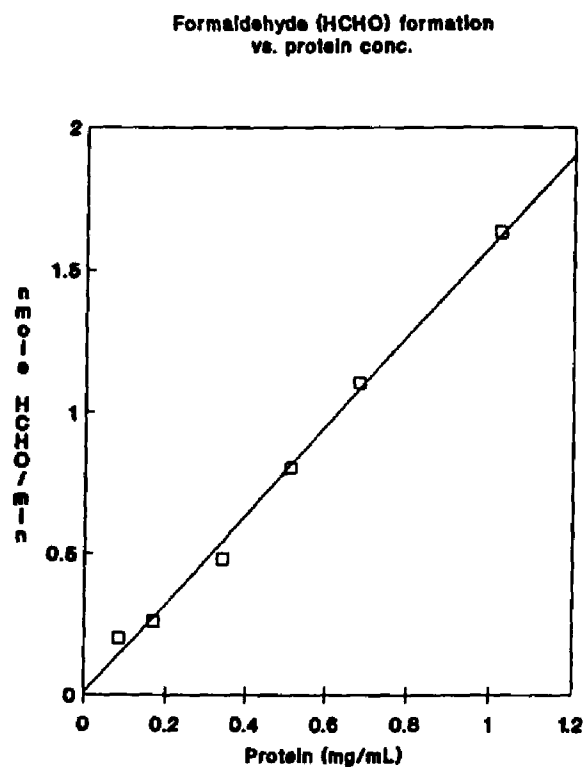
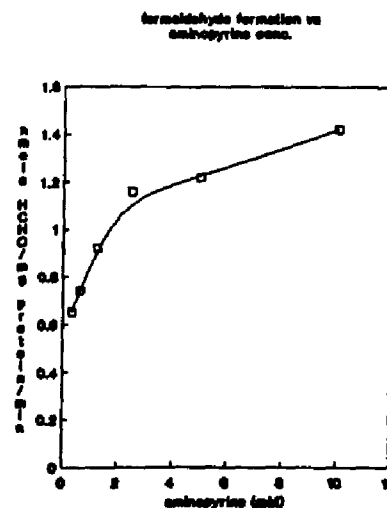
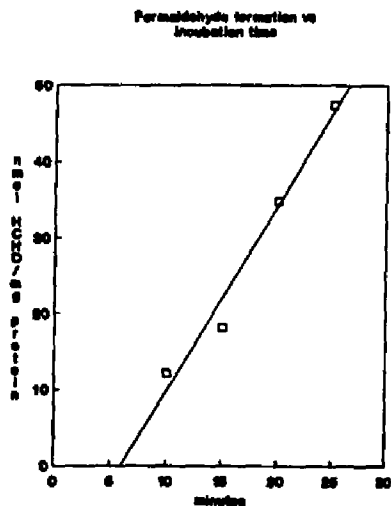
Phosphate conc. vs.
protein conc.



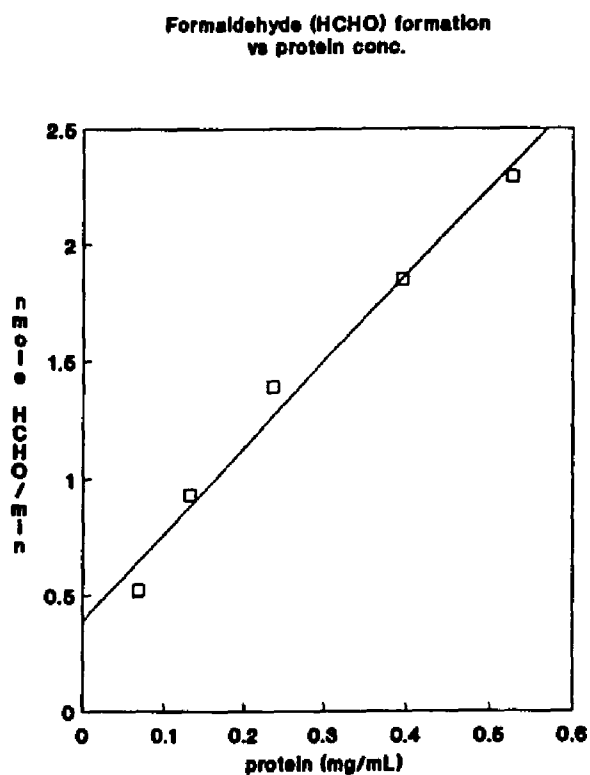
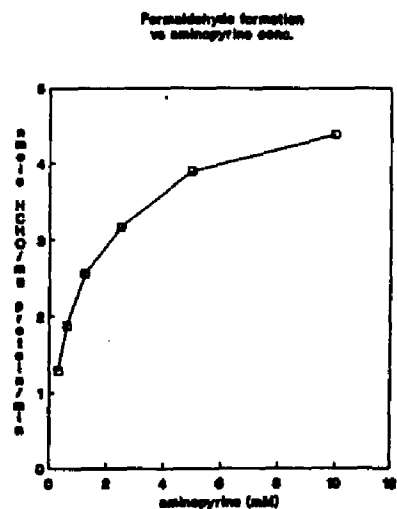
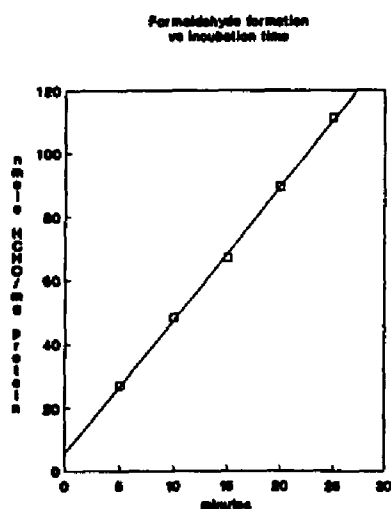
Phosphate conc. vs.
incubation time



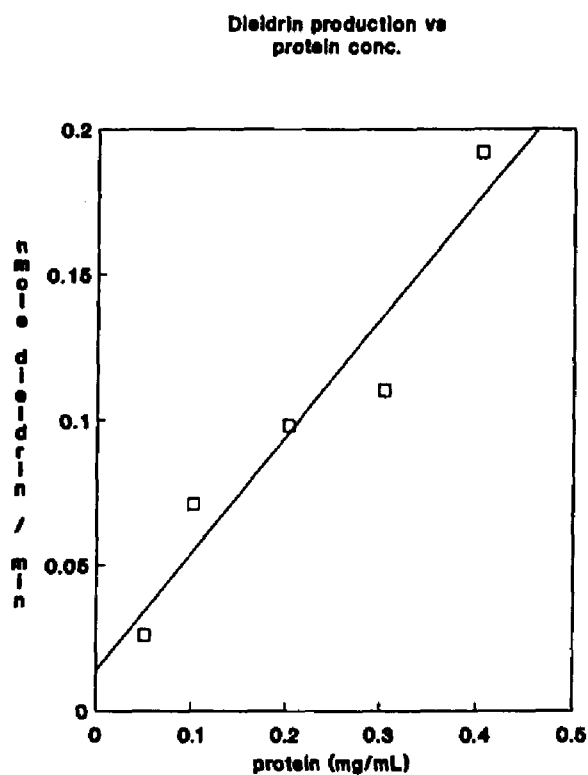
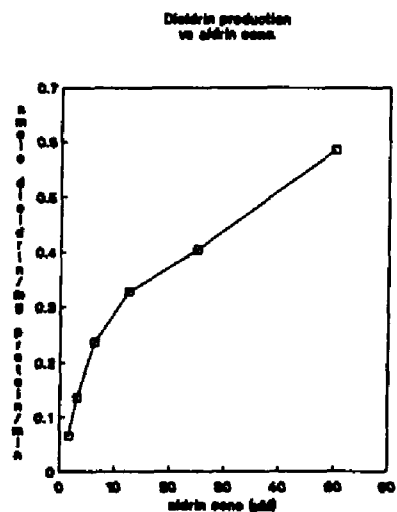
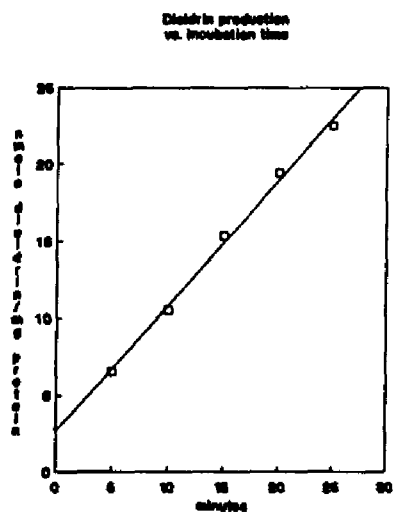
Appendix 10. Phosphate conc. vs. protein conc. and incubation time in the rat hepatic microsomal glucose-6-phosphatase assay.



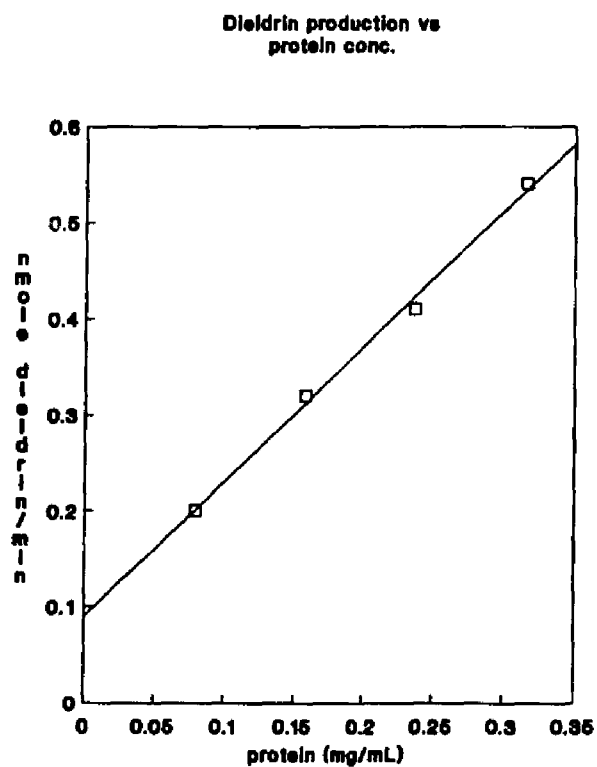
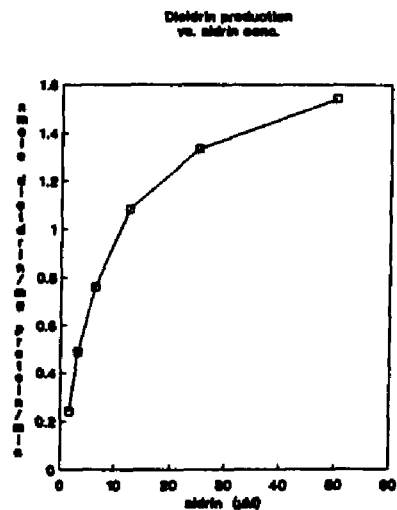
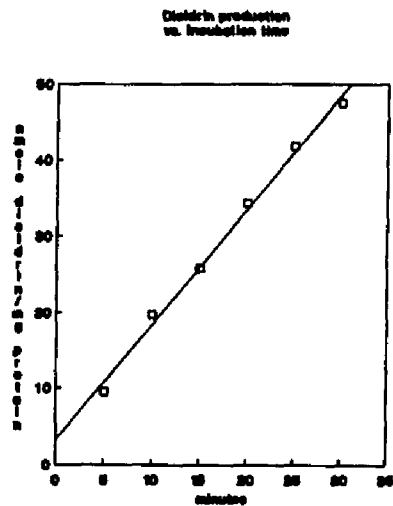
Appendix 11. Formaldehyde formation vs. protein concentration, incubation time, and aminopyrine concentration for the chicken hepatic microsomal aminopyrine N-demethylase assay.



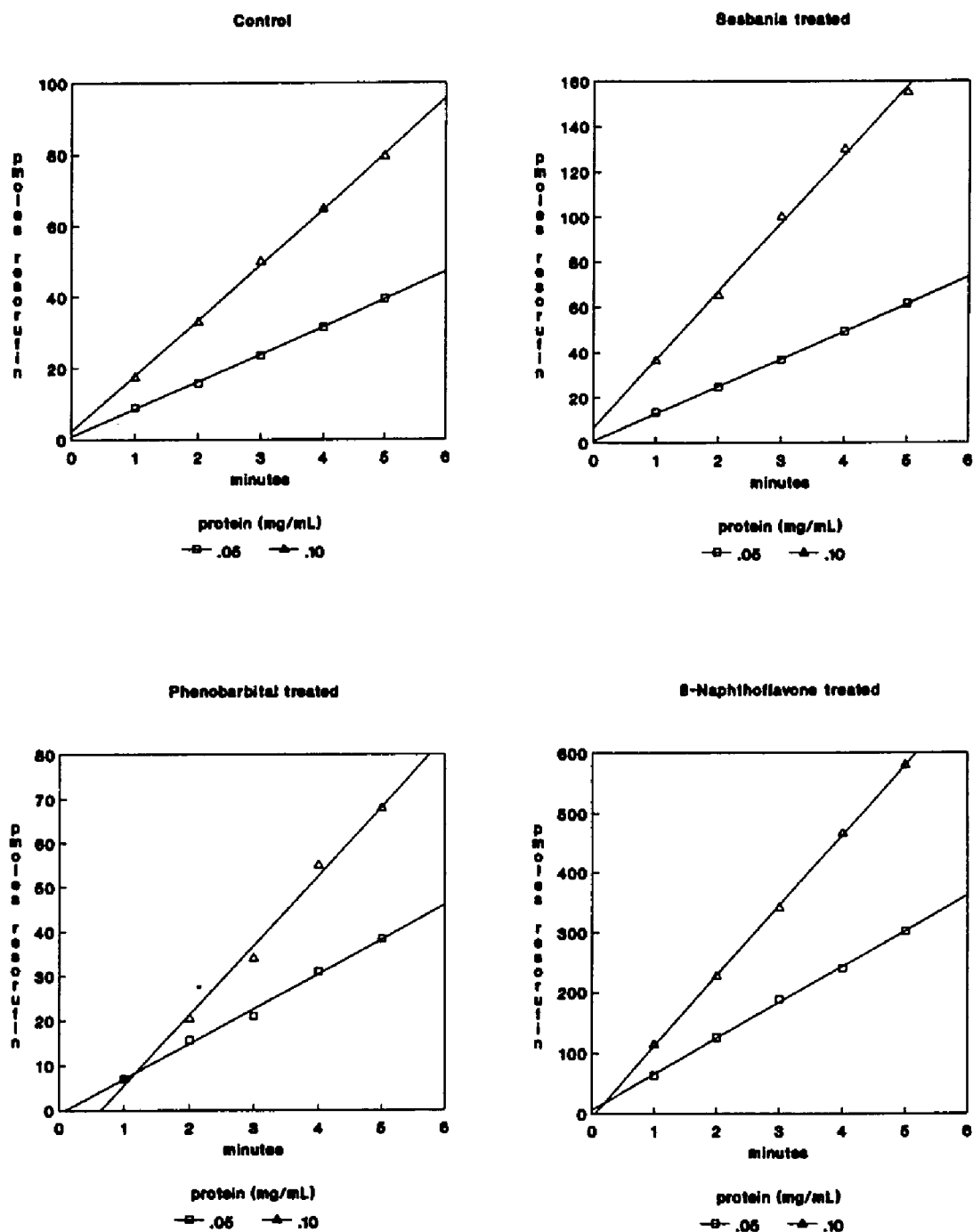
Appendix 12. Formaldehyde formation vs. protein concentration, incubation time, and aminopyrine concentration for the rat hepatic microsomal aminopyrine N-demethylase assay.



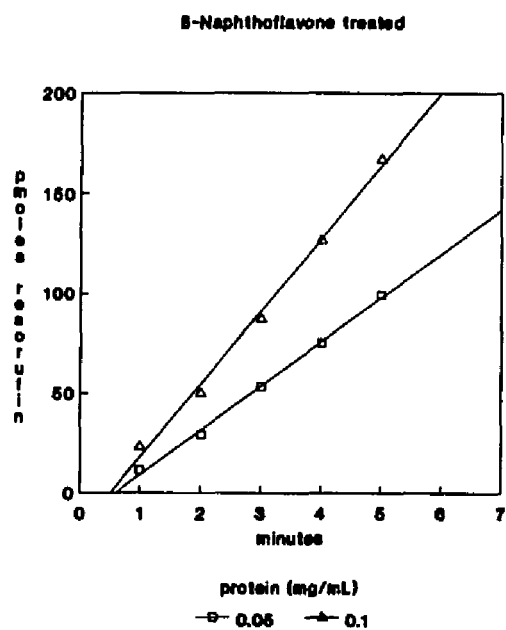
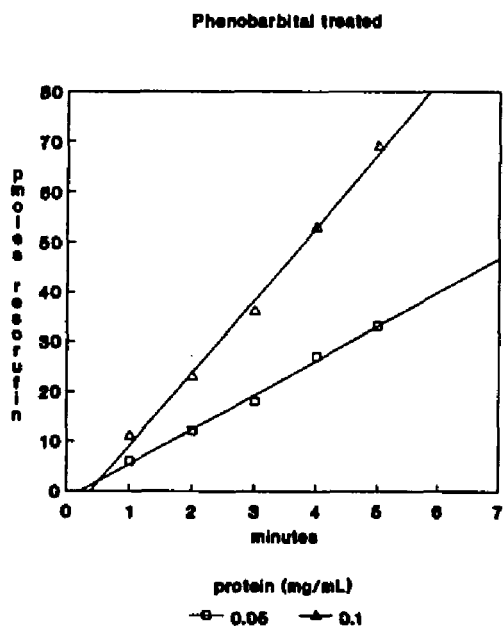
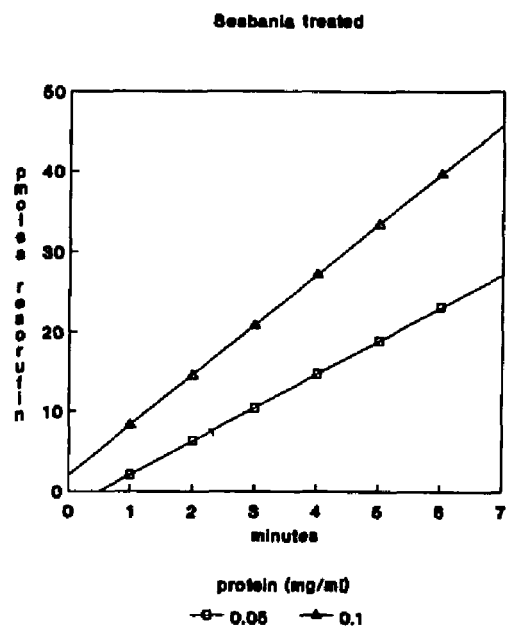
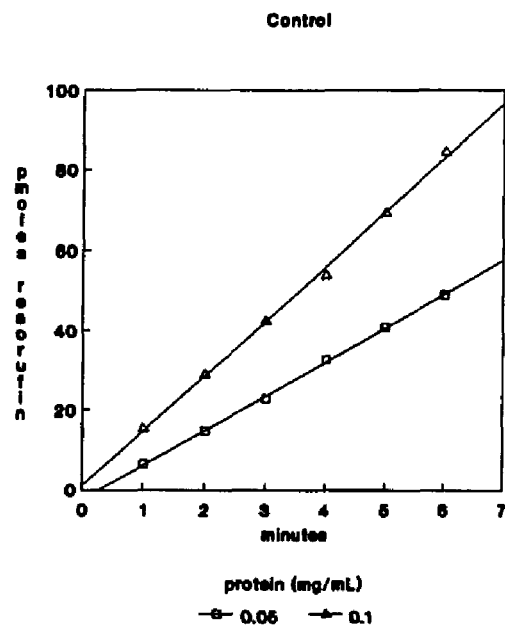
Appendix 13. Dieldrin production vs. protein concentration, incubation time, and aldrin concentration for the chicken microsomal aldrin epoxidase assay.



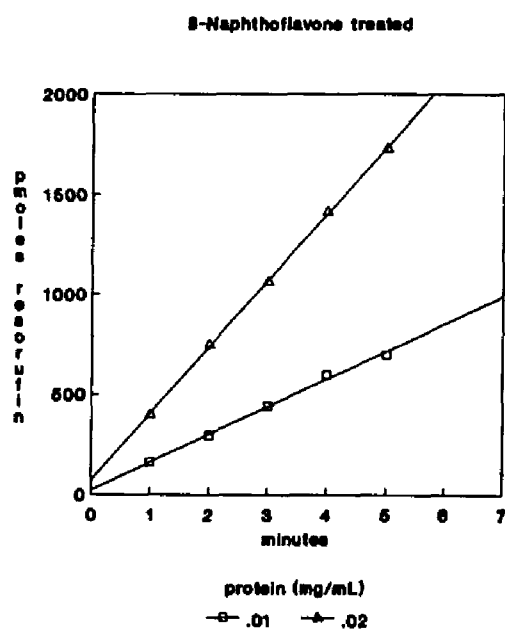
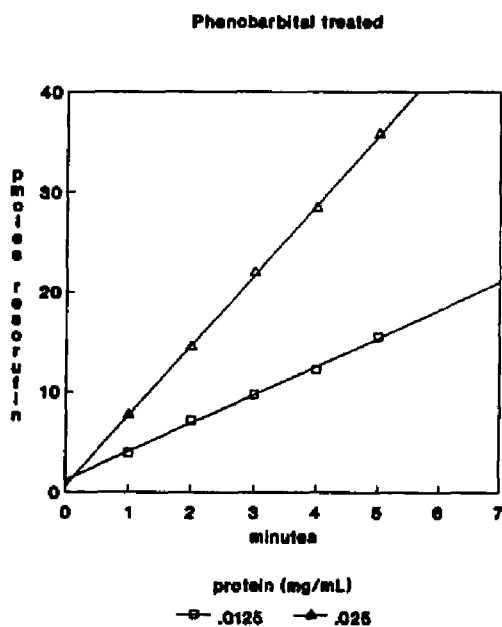
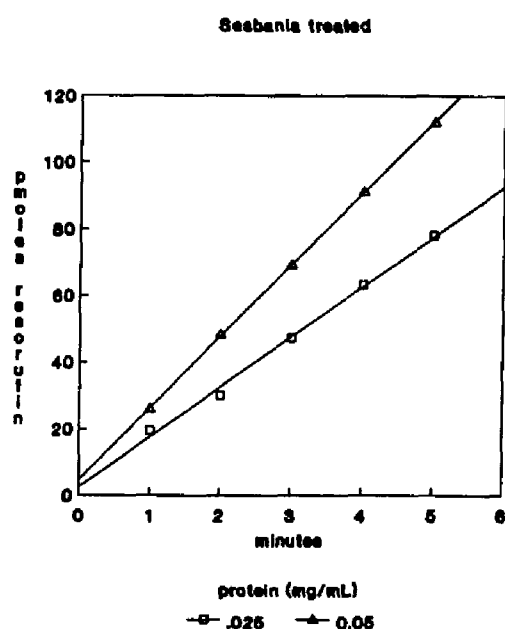
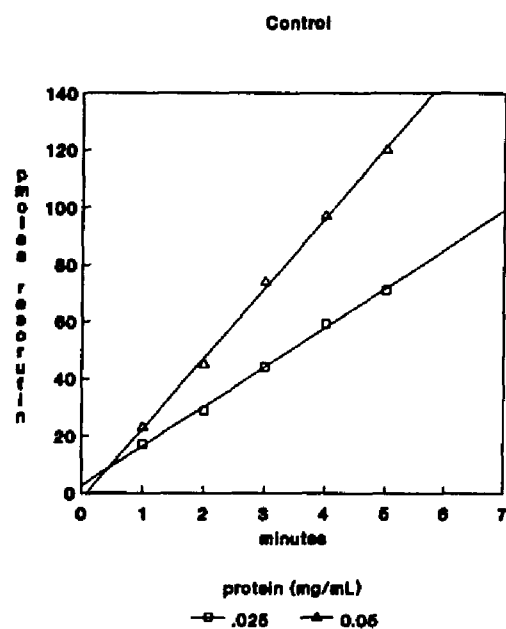
Appendix 14. Dieldrin production vs. protein concentration, incubation time, and aldrin concentration for the rat hepatic microsomal aldrin epoxidase assay.



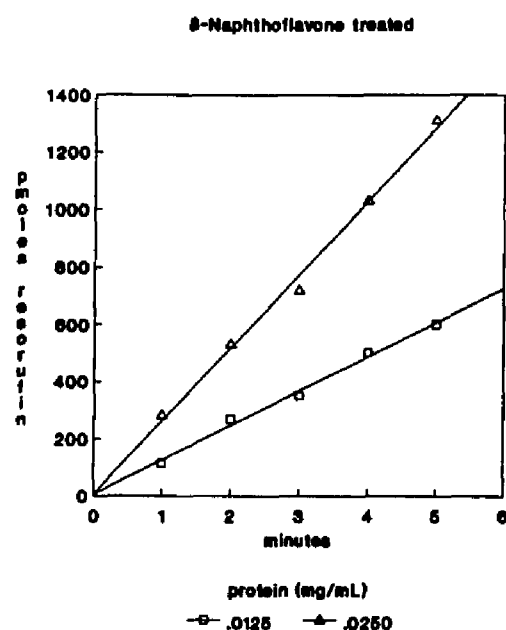
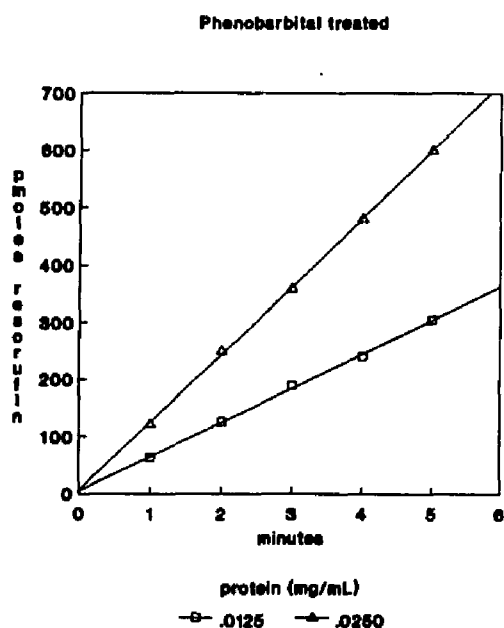
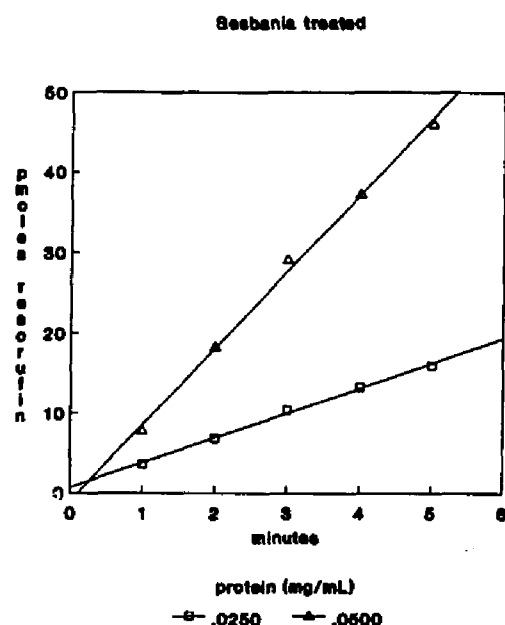
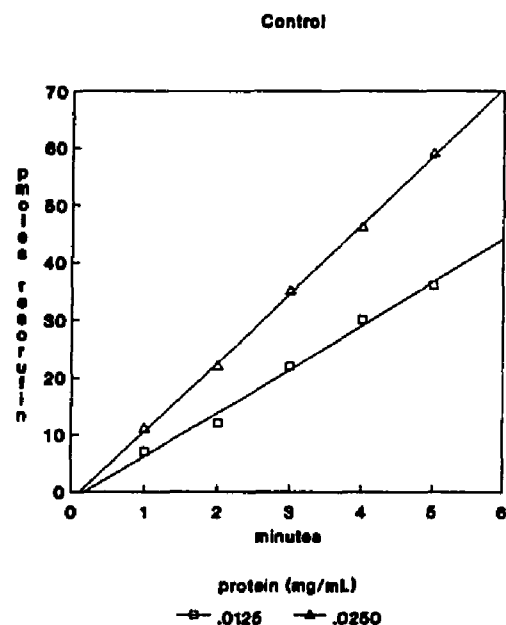
Appendix 15. Resorufin production vs. incubation time at two protein levels in the chicken hepatic microsomal methoxyphenoxazone O-dealkylase assay.



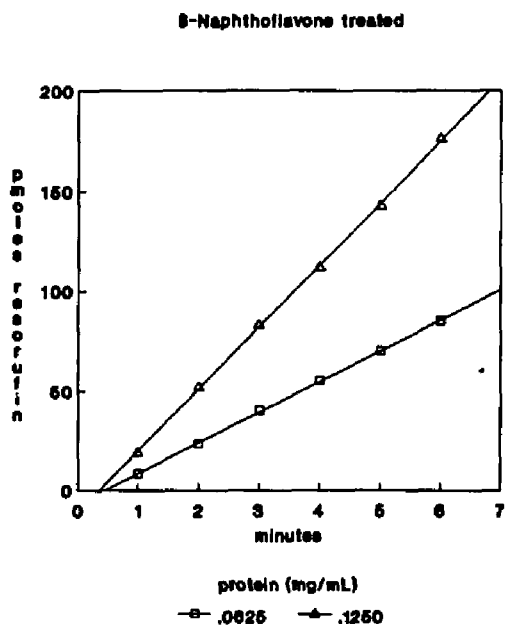
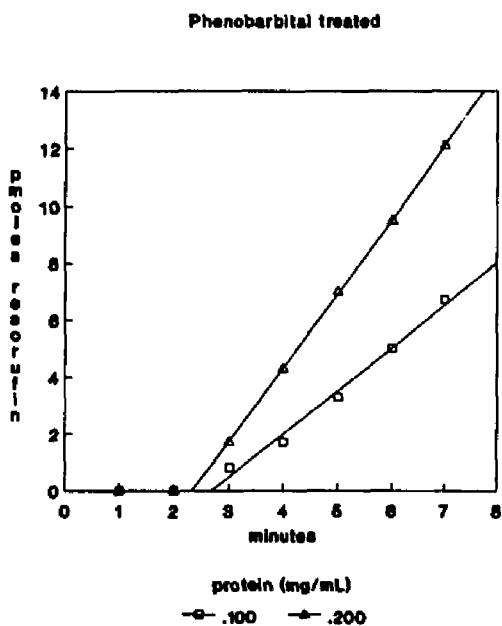
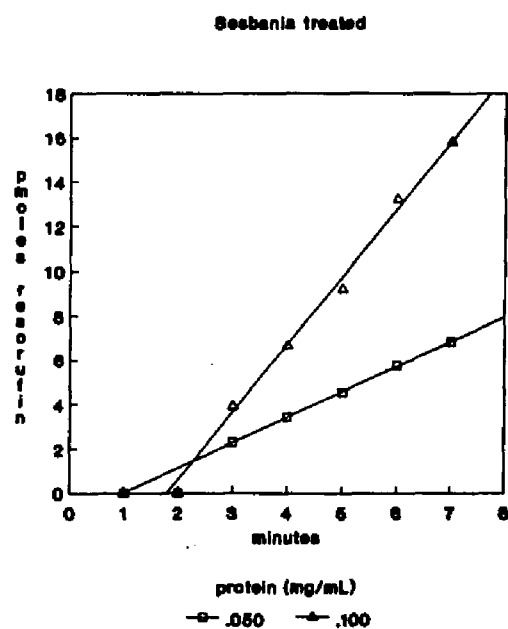
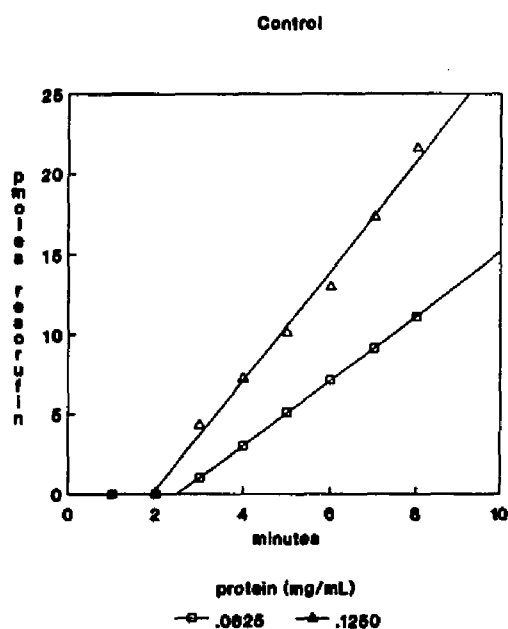
Appendix 16. Resorufin production vs. incubation time at two protein levels in the rat hepatic microsomal methoxyphenoxazone O-dealkylase assay.



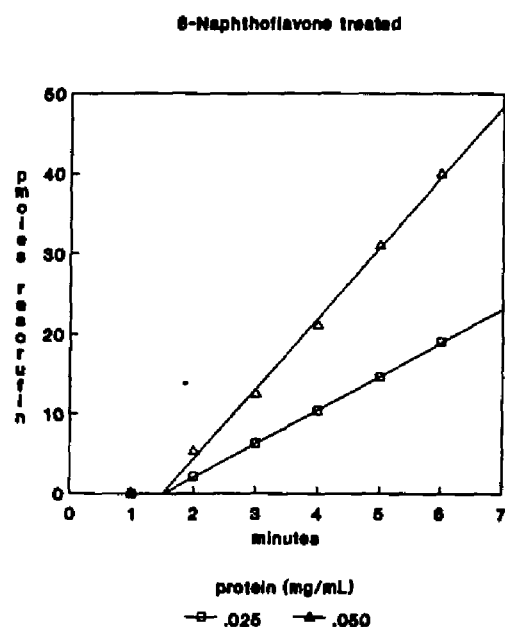
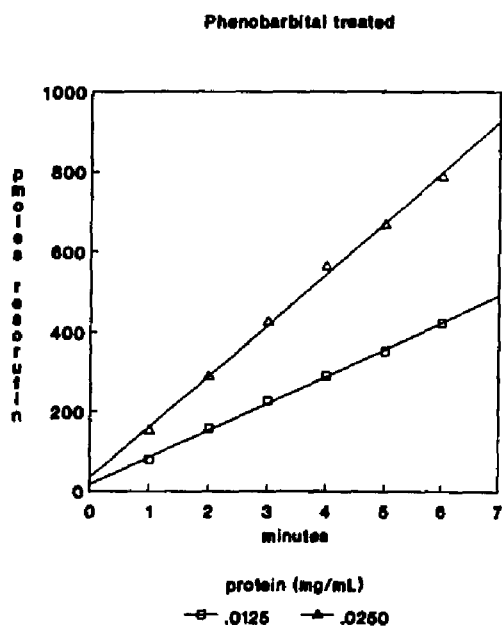
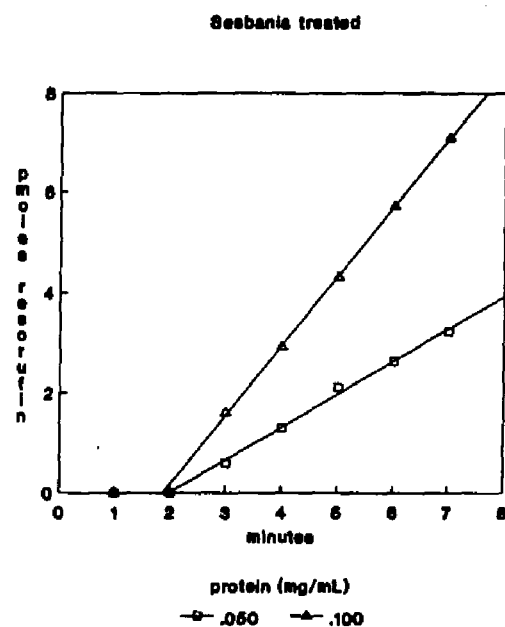
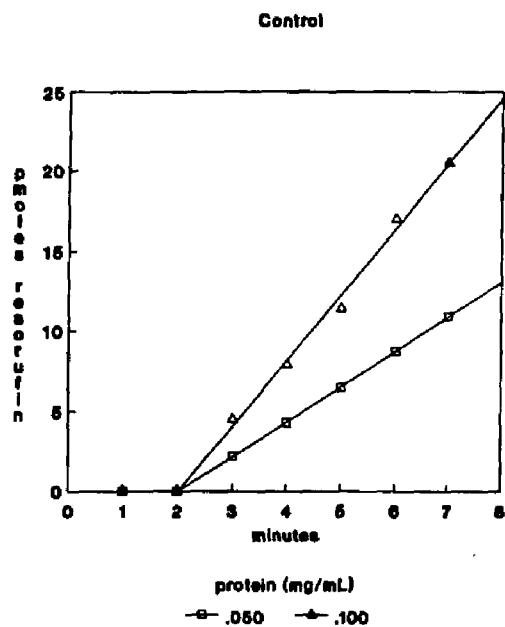
Appendix 17. Resorufin production vs. incubation time at two protein levels in the chicken hepatic microsomal ethoxyphenoxazone O-dealkylase assay.



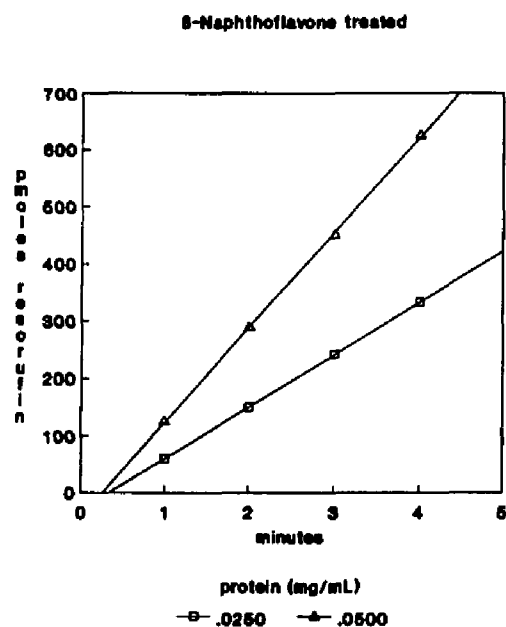
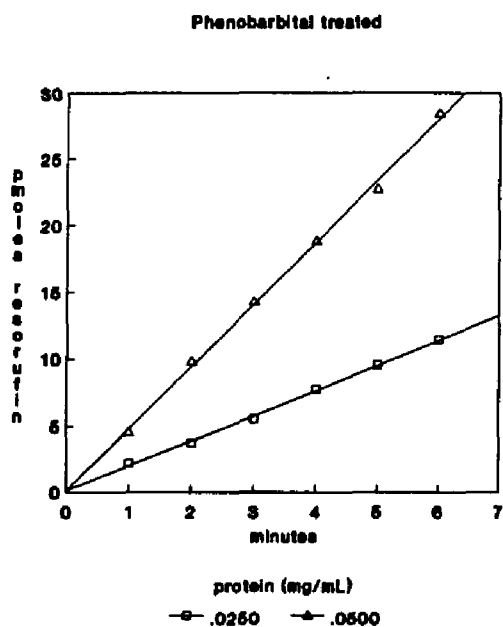
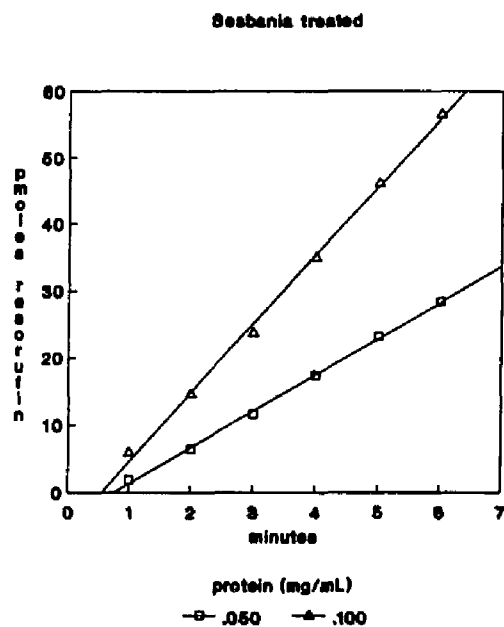
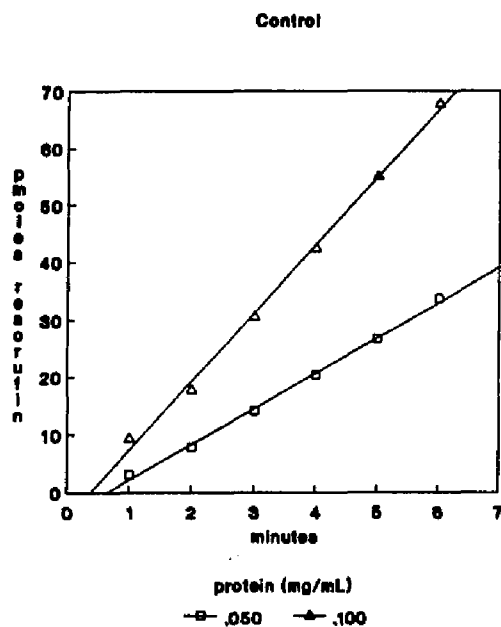
Appendix 18. Resorufin production vs. incubation time at two protein levels in the rat hepatic microsomal ethoxyphenoxazone O-dealkylase assay.



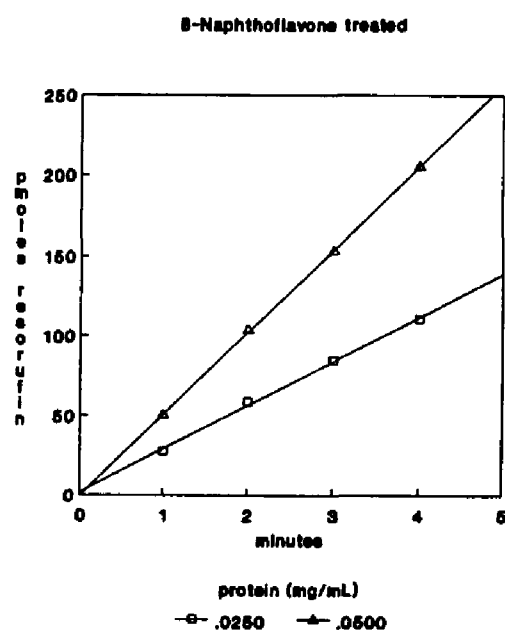
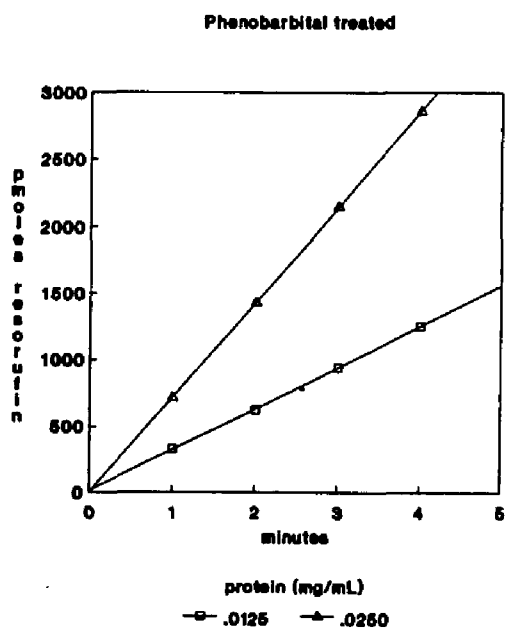
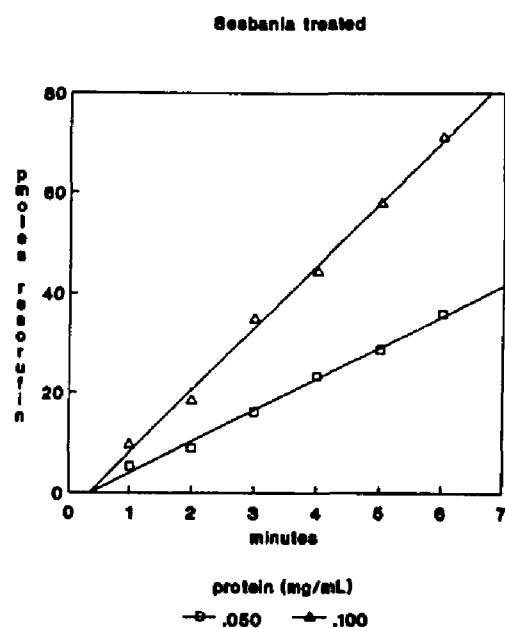
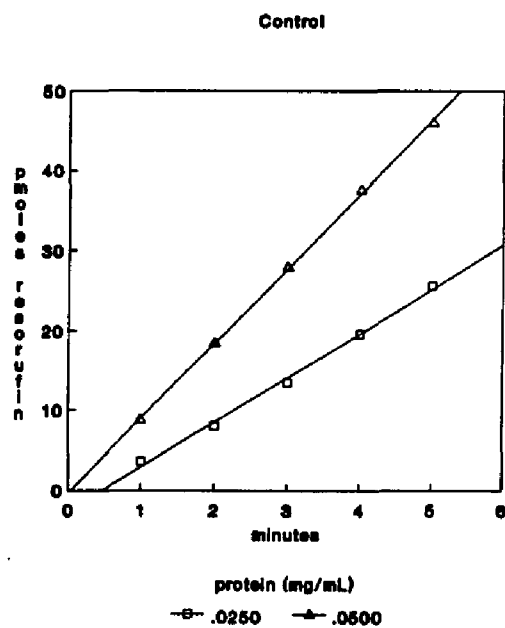
Appendix 19. Resorufin production vs. incubation time at two protein levels in the chicken hepatic microsomal pentoxyphenoxazone O-dealkylase assay.



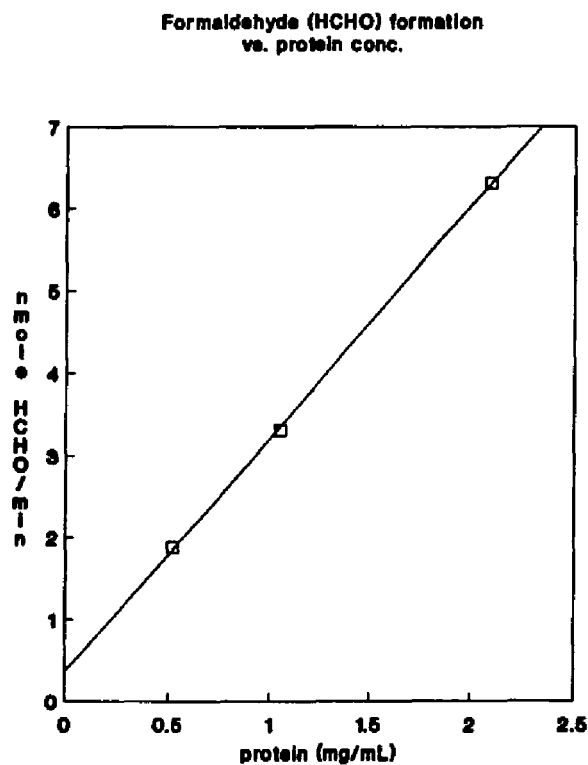
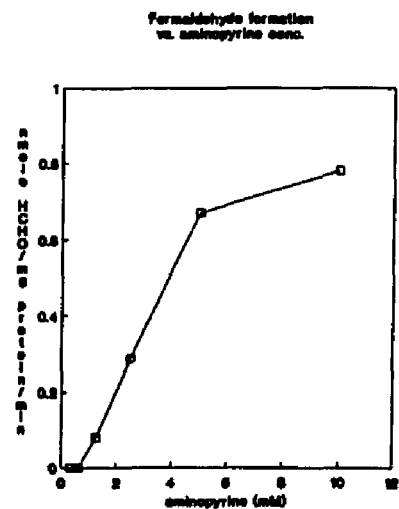
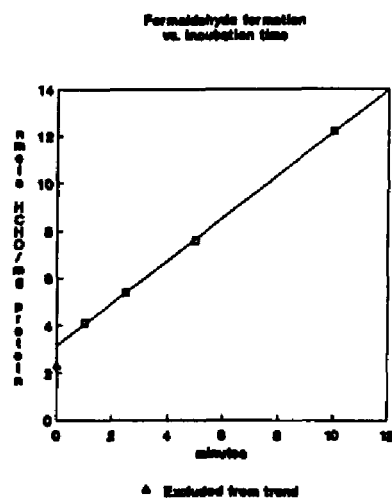
Appendix 20. Resorufin production vs. incubation time at two protein levels in the rat hepatic microsomal pentoxyphenoxazone O-dealkylase assay.



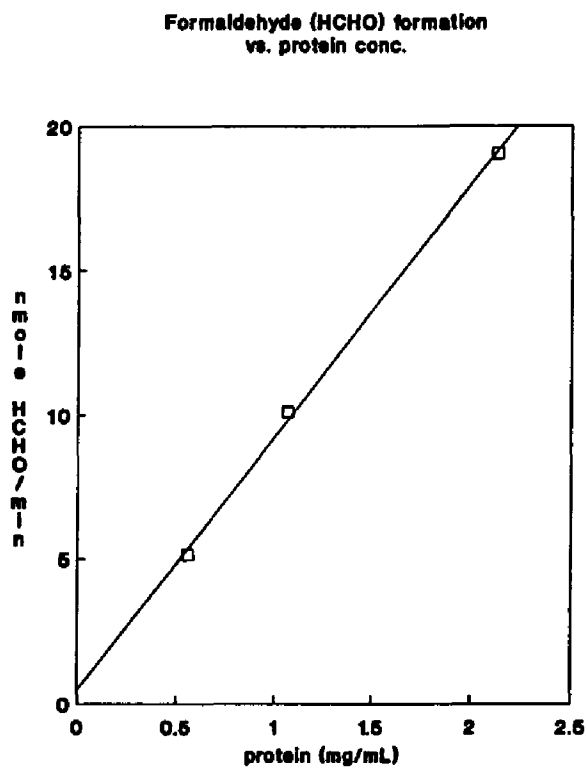
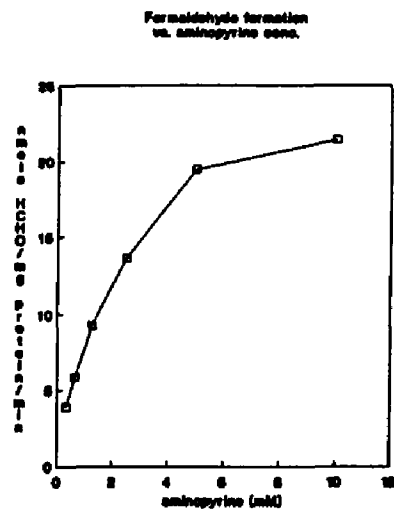
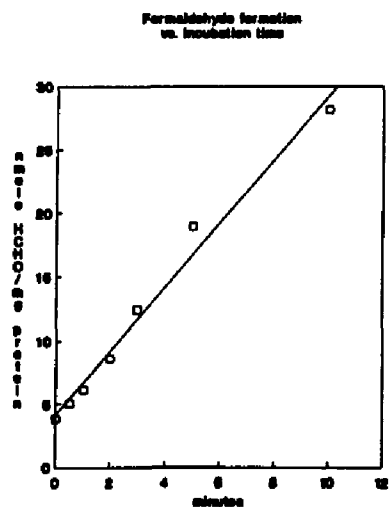
Appendix 21. Resorufin production vs. incubation time at two protein levels in the chicken hepatic microsomal benzyloxyphenoxazone O-dealkylase assay.



Appendix 22. Resorufin production vs. incubation time at two protein levels in the rat hepatic microsomal benzyloxyphenoxazone O-dealkylase assay.

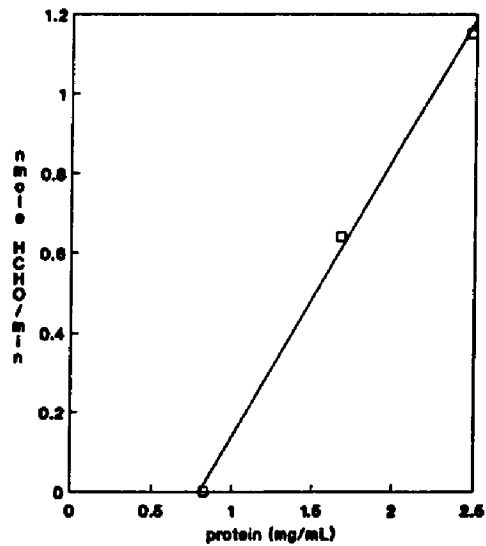


Appendix 23. Formaldehyde formation vs. protein conc., incubation time, and aminopyrine conc. for the chicken hepatic microsomal cumene hydroperoxide-mediated aminopyrine N-demethylase assay

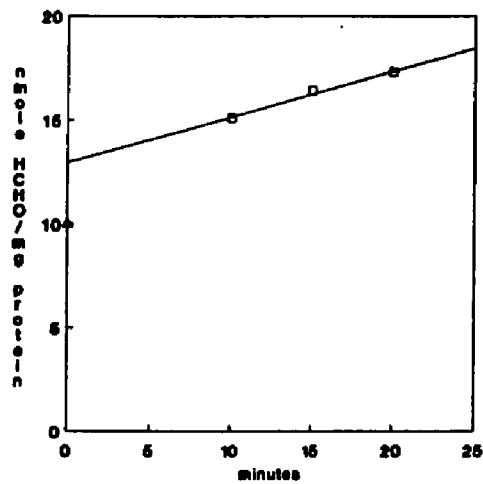


Appendix 24. Formaldehyde formation vs. protein conc., incubation time, and aminopyrine conc. for the rat hepatic microsomal cumene hydroperoxide-mediated aminopyrine N-demethylase assay

Formaldehyde (HCHO) formation
vs. protein conc.



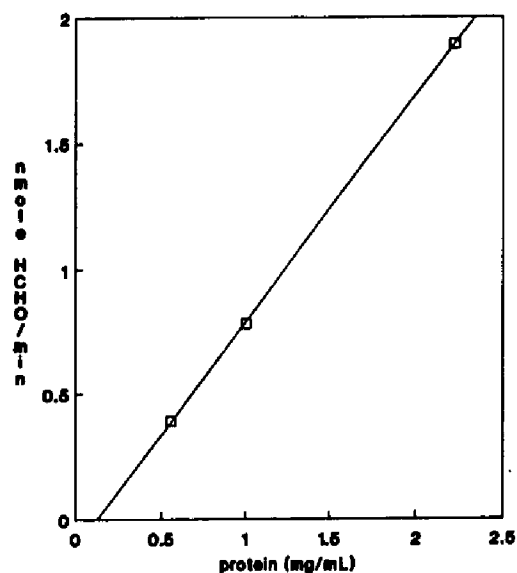
Formaldehyde formation vs
incubation time



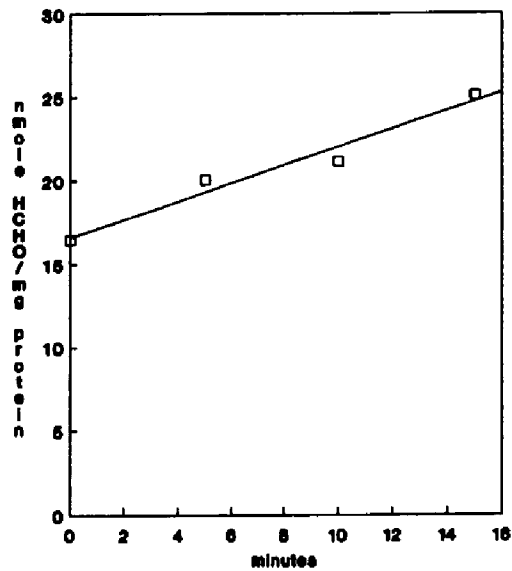
△ Excluded from trend

**Appendix 25. Formaldehyde formation vs. protein conc.,
and incubation time for the chicken hepatic microsomal
t-butyl hydroperoxide-mediated
aminopyrine N-demethylase assay**

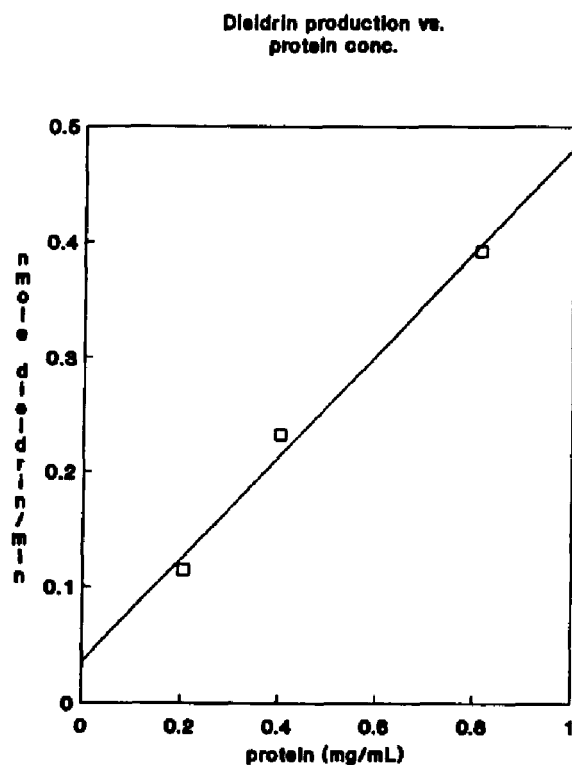
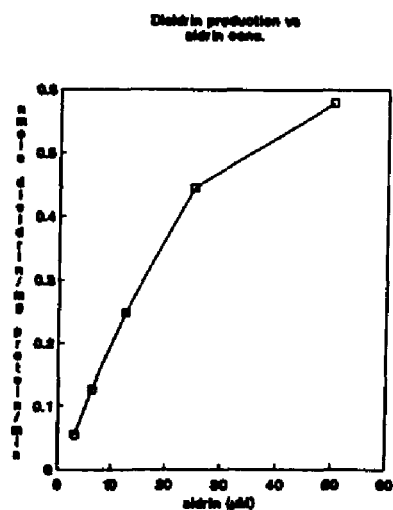
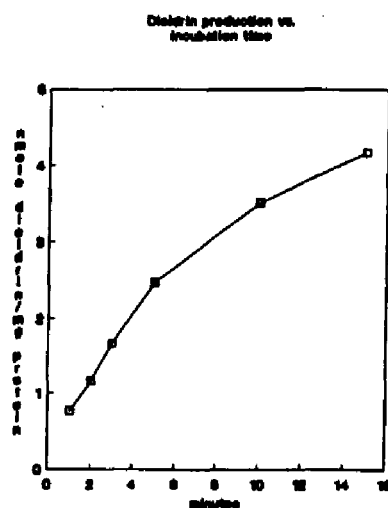
Formaldehyde (HCHO) formation
vs. protein conc.



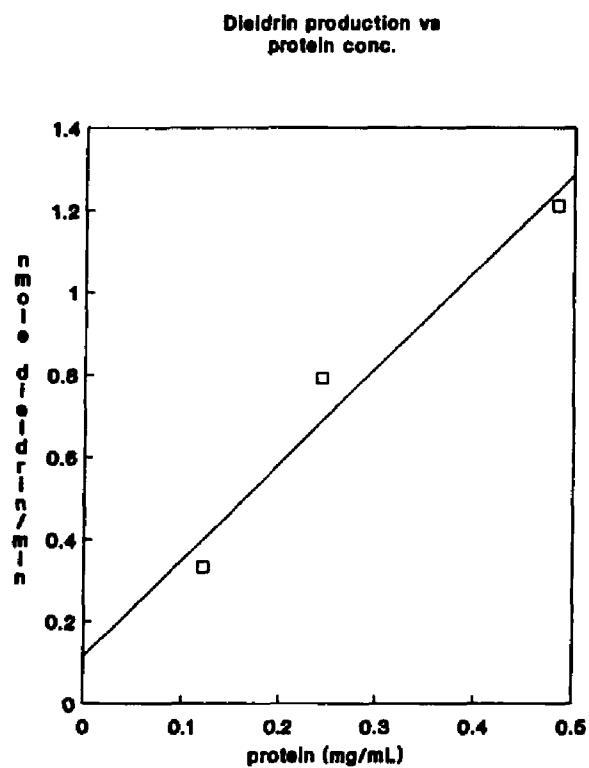
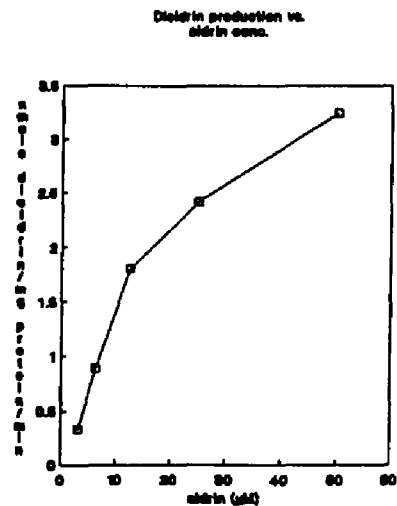
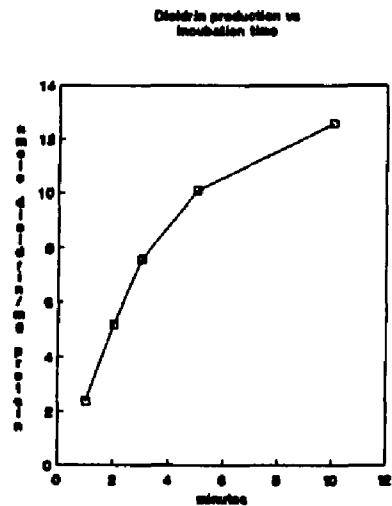
Formaldehyde formation vs
incubation time



Appendix 26. Formaldehyde formation vs. protein conc., and incubation time for the rat hepatic microsomal t-butyl hydroperoxide-mediated aminopyrine N-demethylase assay

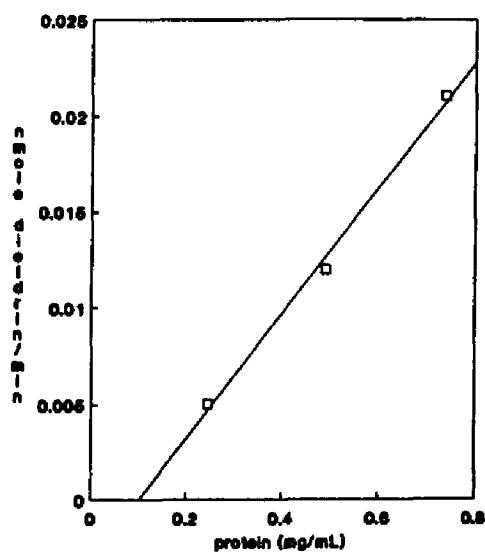


Appendix 27. Dieldrin production vs. protein conc., and incubation time, and aldrin conc. for the chicken hepatic microsomal cumene hydroperoxide-mediated aldrin epoxidase assay.

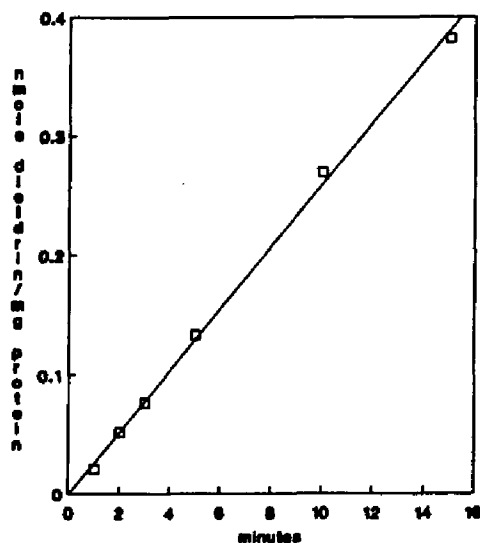


Appendix 28. Dieldrin production vs. protein conc., and incubation time, and aldrin conc. for the rat hepatic microsomal cumene hydroperoxide-mediated aldrin epoxidase assay.

Dieldrin production vs.
protein conc.

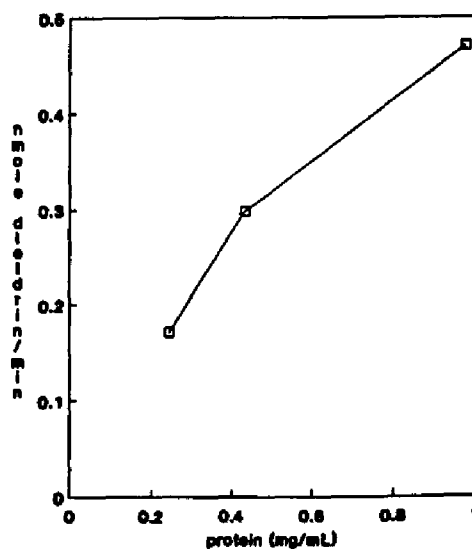


Dieldrin production vs
incubation time

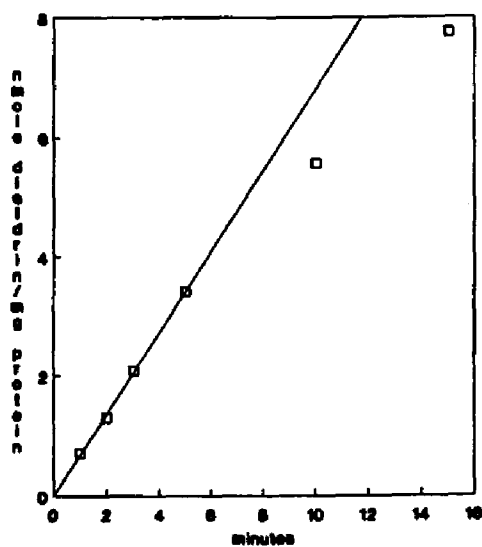


**Appendix 29. Dieldrin formation vs. protein conc.,
and incubation time for the chicken hepatic microsomal
t-butyl hydroperoxide-mediated aldrin epoxidase assay.**

Dieldrin production vs.
protein conc.



Dieldrin production vs
incubation time



**Appendix 30. Dieldrin formation vs. protein conc.,
and incubation time for the rat hepatic microsomal
t-butyl hydroperoxide-mediated aldrin epoxidase assay.**

Appendix 31. Banton, M.I.; Flory, W.; Jowett, P.L.H and Winston, G.W. (1989). Comparison of the effects of Sesbania drummondii on the hepatic microsomal monooxygenase systems of chickens and rats. Am. J. Vet. Res., 50, 1795-1799.

Reprinted from the AMERICAN JOURNAL OF VETERINARY RESEARCH, Vol. 50, No. 10, Pages 1795-1799.
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Comparison of the effects of *Sesbania drummondii* on the hepatic microsomal monooxygenase systems of chickens and rats

Marcy I. Banton, DVM; Wayne Flory, PhD; Peter L. H. Jowett, PhD; Gary W. Winston, PhD

SUMMARY

Sesbania drummondii, a toxic leguminous shrub found throughout the southeastern United States, induces different responses in chicken vs rat hepatic microsomal monooxygenase systems. Groups of 4- to 8-week-old Sprague-Dawley rats and White Leghorn chickens were given extracts of *S drummondii* by gavage for 3 days. Doses, which were 0.4 and 0.8% of daily body weights, respectively, for the rats and chickens, were adjusted to induce similar clinical lesions in the 2 species. The hepatic microsomal monooxygenase systems of control and treated animals were compared, using cytochrome P-450 content, cytochrome *b₅* content, NADH- and NADPH-cytochrome *c*-reductase activity, and 6 cytochrome P-450 mediated enzyme activities. Increases of twofold in the cytochrome P-450 content, NADPH-cytochrome *c*-reductase, aminopyrine-*N*-demethylase, aniline hydroxylase, ethoxycoumarin-*O*-deethylase, and aryl hydrocarbon hydroxylase activities; fourfold in the aldrin epoxidase activity; and 15-fold in the ethoxycoumarin-*O*-deethylase activity were observed in the *S drummondii*-treated chickens. In contrast, the treated rats had nearly twofold decreases in these values, suggesting a species-specific effect of *S drummondii* on microsomal monooxygenase systems, is, induced with *S drummondii*.

Sesbania drummondii is a perennial herbaceous shrub or small tree inhabiting the sandy soils and marshes throughout southeastern United States. This poisonous plant, also called rattlebrush, coffeebean, or rattlebox, is sporadically ingested by livestock and usually results in fatalities.¹ Poisonings have been reported in cattle, sheep,

and goats, usually in the fall or winter after other forage has become scarce.^{2,3} The toxic principle of *S drummondii* has not yet been identified, but appears to be concentrated in the mature seed-containing pods.⁴

Chickens and rats have been proven experimentally to be highly sensitive to extracts of *S drummondii* seeds and are, therefore, useful avian and mammalian experimental models for studying the toxic effects of the plant.⁵ Clinical signs, blood chemistry values, and tissue lesions have been previously described in poisoned chickens.⁶ Typically, the clinical signs include weakness, depression, anorexia, diarrhea, ruffled feathers, cold feet, and rapid loss of body weight. An interesting clinical feature in chickens has been a severe decrease in the concentration of total plasma proteins.⁷ At necropsy, gross changes are evident in body fat, muscle mass, liver, kidneys, spleen, crop, intestines, and the heart. Similar clinical and pathologic changes are observed in rats.⁸

Some of the changes in poisoned birds, as well as in rats, can be explained by inhibition of smooth muscle function.⁹ However, other changes in both species may be caused by toxic effects of *S drummondii* on the liver. Earlier studies have focused on the liver to determine the cause of depleted circulating serum protein concentrations.⁸ These results indicated that *S drummondii* inhibits protein synthesis and export protein release from the liver.⁸ Further investigation revealed that *S drummondii* also alters a number of liver enzymes, including microsomal glutathione-S-transferase isozymes.⁸

The purposes of the study reported here were to characterize and compare the effects of *S drummondii* on the major biotransformation system, the hepatic microsomal monooxygenase system, in susceptible avian and mammalian species, namely chickens and rats.

Materials and Methods

Animals.—Twenty-four male and female 300- to 500-g White Leghorn chickens¹⁰ were group-housed in commercial brooders. Eighteen male Sprague-Dawley 200- to 300-g rats were maintained 3/age on a 12-hour light/dark cycle in a temperature- and humidity-controlled environment. Chickens and rats were given feed (standard commercial diets) and water ad libitum. Both species were acclimated for 1 week. Experiments using

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Supported in part by a Hatch grant No. LA-2286 (Flory) and a faculty pass through grant from the Louisiana State University Center for Energy Studies (Winston).

The authors thank Dr. Soren Mørbye for technical assistance in the performance of the assays.

Address reprint requests to Dr. Flory.

* Marum-Dry, M-L. A study on the toxicity of *Sesbania drummondii* in chickens and rats. PhD thesis, Louisiana State University, Baton Rouge, 1988.

¹⁰ White Leghorn chickens obtained from the Department of Veterinary Science, Louisiana State University, Baton Rouge.

chickens were performed with 3 groups of 8 animals, 4 treated and 4 control, in each group. Experiments using rats were performed with 3 groups of 6 animals, 3 treated and 3 control, in each group.

Treatments—Extracts of *S. drummondii* were prepared from dry ground seeds and pods gathered locally. Ground seeds and pods have virtually the same toxicity as whole ground seeds.² The ground seeds and pods were soaked overnight in a 25 mM sodium bicarbonate buffer (pH 7.4) containing 0.02% sodium azide in a ratio of 1 part ground seeds to 5 parts buffer. Sodium azide was added to inhibit bacterial growth in the extract. The extract was recovered by vacuum filtration through 6 layers of cheese cloth. The concentration of the extract was determined from the volume of filtrate per gram of seeds and pods. Animals were weighed daily for 3 days and then either were given freshly prepared extract of *S. drummondii* by gavage or received no treatment. Control animals were not given the extracts. Differences in the growth, health, microsomal content or activities of control chickens and rats treated with the bicarbonate-azide buffer vs those not given the buffer have not been observed in previous experiments in our laboratory. The volume of extract given was calculated by multiplying the body weight of the animal, the dose as percentage of body weight, and the concentration of extract. Treated chickens received daily doses equal to 0.8% of body weight, and rats received 0.4% of body weight. These doses have been found to induce similar clinical signs and necropsy lesions in the 2 specimens over a 3-day period.²

Preparation of microsomes—On the fourth day, the animals were weighed, killed by guillotine, and examined for gross lesions. Livers were carefully removed, weighed, and placed on ice. Microsomes were prepared from the pooled livers of the group, treated or control. The livers were minced, rinsed, and homogenized in 4 volumes of ice-cold buffer solution (10 mM tris, pH 7.4; 0.25M sucrose; and 1.0 mM disodium EDTA). A microsomal pellet was prepared by stepwise differential centrifugation of the homogenate³ at $600 \times g$, $8,000 \times g$, and $14,500 \times g$, and subsequent centrifugation of the supernatant at $105,000 \times g$. The resulting pellet was washed once with 0.125M KCl and recentrifuged at $105,000 \times g$ for 1 hour. The final microsomal pellet was resuspended in 0.125M KCl in 20% glycerol and frozen (-70°C) until used. Microsomes were used within 2 weeks of isolation. The concentration of microsomal proteins was determined, using bovine serum albumin as a reference standard.⁴

Determination of cytochrome content and reductase activities—Cytochrome P-450 content of the microsomes was determined by the method of Omura and Sato,⁵ using the difference spectrum of the carbon monoxide complex of dithionite-reduced cytochrome P-450 minus the spectral contribution of reduced cytochrome P-450. The change in absorbance at 450 nm relative to 490 nm was then converted to the specific content of cytochrome P-450, using an extinction coefficient of $91 \text{ cm}^{-1}\text{mM}^{-1}$. The concentration of cytochrome b₅ in the microsomal suspensions was determined from the NADH-reduced difference spectrum by using the extinction coefficient of $185 \text{ cm}^{-1}\text{mM}^{-1}$ for the absorbance change at 426 minus 409 nm.⁶

The NADH and NADPH-cytochrome c-reductase activities were assayed by use of the method of Phillips and Langdon,⁷ as modified by Yasukochi and Masters,¹¹ in which the rate of cytochrome c reduction was followed spectrophotometrically at 550 nm. The reductase activities were quantified, using an extinction coefficient of $19.5 \text{ nm}^{-1} \text{ cm}^{-1}$ for the reduced minus the oxidized forms.

Measurement of enzyme activities—The microsomal-mediated metabolism of aminopyrine was measured by analysis of the formation of formaldehyde. The reaction mixture consisted of 0.4 mg of microsomal protein, 0.3 mM NADP⁺, 10 mM glucose-

6-phosphate, 7 U of glucose-6-phosphate dehydrogenase, 10 mM MgCl_2 , 100 mM potassium phosphate buffer (pH 7.4), and 10 mM aminopyrine in a final volume of 3.0 ml. The reaction was started by the addition of the NADPH generating system, incubated at 37°C for 15 minutes, and stopped by the addition of 1 ml of 70% trichloroacetic acid. Aminopyrine-N-demethylase (APND) was quantitated by measuring the amount of formaldehyde formed according to the method of Nash,¹² compared with a standard curve prepared in the reaction matrix.

Aniline hydroxylase activity (ANH) was measured by following the formation of p-aminophenol from aniline according to the method of Lake,⁷ with modifications. The 2-ml reaction mixture contained 1 mg of microsomal protein, 50 mM tris-HCl buffer (pH 7.8), 5 mM MgSO_4 , 0.5 mM NADP⁺, 5 mM glucose-6-phosphate, 1.5 U of glucose-6-phosphate dehydrogenase, and 5 mM aniline-HCl. The reaction was started by the addition of the NADPH generating system and was incubated in a 37°C water bath for 10 minutes. The reaction was terminated by adding 2 ml of 10% trichloroacetic acid. After centrifugation, a 2-ml aliquot of the supernatant was added to 1 ml of 10% Na_2CO_3 , followed by the addition of 2 ml of 1% phenol in 0.1N NaOH. The resulting optical density was measured at 620 nm after 30 minutes and compared with a p-aminophenol standard.

Aldrin epoxidase activity (AE) was determined from the amount of dieldrin produced, as described by Riviere et al.¹³ with modifications. The incubation system contained 50 mM potassium phosphate buffer (pH 6.8), 0.25 mM NADP⁺, 2.5 mM glucose-6-phosphate, 1 U of glucose-6-phosphate dehydrogenase, 0.2 mg of microsomal protein, and 50 μM aldrin¹⁴ in a final volume of 1.0 ml. Incubation was performed aerobically in a constant temperature shaker at 37°C for 15 minutes. The reaction was initiated by the addition of aldrin and stopped by the addition of 0.2 ml of acetone. Hexane (5 ml) was added, and the tubes were capped and vortexed for 45 seconds. Dieldrin¹⁵ was assayed by use of a gas chromatograph¹⁶ equipped with an electron-capture detector (⁶³Ni) and a capillary column¹⁷ (15 m long, 0.53 mm ID, phase 007-17). The column, detector, and injector temperatures were 220 to 340°C ($10^\circ\text{C}/\text{min}$), 300°C , and 180°C , respectively. The injection of samples was made in the splitless mode, using a direct flask injection liner. The carrier gas was 5% methane in argon at a flow rate of 11 ml/min. Carrier gas was also used for detector make-up to a total flow of approximately 30 ml/min. The amount of dieldrin in the extract was quantified by peak height from a standard curve.

The O-dealkylation of 7-ethoxycoumarin and 7-ethoxyresorufin¹⁸ was measured fluorometrically using a spectrophotofluorometer,¹⁹ by the method of Lake.⁷ The 7-ethoxycoumarin-O-deethylase (ECOD) assay was run at 37°C for 10 minutes and contained a standard reaction mixture of 50 mM tris HCl buffer (pH 7.8), 5 mM MgSO_4 , 0.5 mM NADP⁺, 5 mM glucose-6-phosphate, 3 U of glucose-6-phosphate dehydrogenase, 0.5 mM 7-ethoxycoumarin, and 1 mg of microsomal protein in a final volume of 2 ml. The 7-ethoxyresorufin-O-deethylase (EROD) assay was also performed at 37°C for 10 minutes and contained the aforementioned components in the reaction mixture, with the exception that 5 μM 7-ethoxyresorufin was used for the substrate and that the microsomal protein concentration was 0.1 mg/ml. The buffer used was 50 mM tris HCl (pH 8.4), instead of the tris HCl (pH 7.8). The reactions were started with the addition of substrate and terminated by the addition of 1 ml of zinc sulfate solution, followed by 1 ml of saturated barium hydroxide solution to all tubes. After centrifugation, 1.5 ml of supernatant was removed and added to 3 ml of 0.5M glycine-sodium hydroxide buffer (pH 10.5 for ECOD, pH 8.5 for EROD). The ECOD assay used 380-nm excitation and 452-nm emission

* EPA, Research Triangle Park, NC.

¹⁴ Hewlett Packard 5890 A chromatograph, Hewlett Packard, Avondale, Pa.

¹⁵ Quadron Corp., New Haven, Ct.

¹⁶ Sigma Chemical Co., St. Louis, Mo.

¹⁷ Parread Optical Co., Valhalla, NY.

wavelengths and was compared with an umbelliferone (7-hydroxycoumarin) standard. The ECOD activity was measured, using 535 nm excitation and 582-nm emission wavelengths and was quantitated with 7-hydroxyresorufin as a standard.

The aryl hydrocarbon hydroxylase activity (AHH) was measured by the method described by Guengerich.¹⁴ The components of a 1 ml reaction mixture included 50 mM potassium phosphate buffer (pH 7.4), 5 mM $MgCl_2$, 1 mM $NADP^+$, 10 mM glucose-6-phosphate, 3 U of glucose-6-phosphate dehydrogenase, 80 μM benzo[a]pyrene,¹ and 0.1 mg of microsomal protein. The reaction was started with the NADPH generating system. Incubations were performed at 37°C for 10 minutes and were terminated with 1 ml of cold acetone (4°C). The products were extracted with 3.25 ml of hexane followed by the addition of 1N NaOH. Fluorescence of the aqueous layer was read with the excitation at 396 nm and the emission at 522 nm. The aryl hydrocarbon hydroxylase activity was quantitated, using a 3-OH benzo[a]pyrene standard.

Statistical analysis—The significance of difference between nontreated controls and treated groups was determined, using the Student *t* test. The *P* values > 0.05 were not considered statistically significant.

Results

Body weights and liver weights—Decreases in body weights in chickens were detected in the *S. drummondii*-treated groups, compared with controls. Weight losses developed, despite detection of large quantities of food material in the crops of treated chickens at necropsy. The treated rats had slight, but not significant, decreases in gains in body weight relative to the controls. Liver weight changes paralleled those of body weight in the treated animals, resulting in liver to body weight percentages similar to those of the control animals (Table 1).

Hepatic microsomal cytochromes and reductases—Striking differences in the specific content of microsomal cytochrome P-450 of *S. drummondii*-treated vs control animals were detected (Table 2). An approximate twofold increase in chickens ($P < 0.01$) and a twofold decrease in rats ($P < 0.05$) cytochromes P-450 were consistently observed for the treated groups. Significant differences were not observed in the cytochrome *b*₅ content of microsomes from *S. drummondii*-treated chickens or rats.

The effect of *S. drummondii* treatment on NADPH- and NADH-cytochrome *c*-reductase activities followed a pattern similar to that described for the cytochromes. Marked increases in the chickens ($P < 0.01$) and slight, but not significant, decreases in the rats were observed for NADPH-

TABLE 2—Effect of *Sesbania drummondii* treatment on hepatic microsomal monooxygenase cytochromes and reductases in chickens and rats

	Chickens		Rats	
	Control	Treated	Control	Treated
Cytochrome P-450 (nmol/mg of protein)	0.17 ± 0.04	0.36 ± 0.07*	0.71 ± 0.06	0.43 ± 0.09*
Cytochrome <i>b</i> ₅ (nmol/mg of protein)	0.11 ± 0.04	0.18 ± 0.04	0.21 ± 0.04	0.23 ± 0.07
NADH cytochrome <i>c</i> -reductase (nmol reduced/min/mg of protein)	160 ± 36	178 ± 36	807 ± 18	535 ± 102
NADPH cytochrome <i>c</i> -reductase (nmol reduced/min/mg of protein)	64 ± 16	156 ± 12*	99 ± 26	64 ± 23

Data are expressed as group means (± SEM; n = 3). Significantly different (Student *t* test) from control data (* $P < 0.01$, * $P < 0.05$).

cytochrome *c*-reductase activity. Significant differences were not observed for NADH-cytochrome *c*-reductase activity in both species treated with *S. drummondii*.

Hepatic microsomal monooxygenase activities—The effects of *S. drummondii* treatment on 6 selected hepatic microsomal monooxygenase enzyme activities are compared in terms of milligrams of microsomal protein (specific activity) and nanomoles of cytochrome P-450 (molar activity or turnover; Table 3).

When expressed as per milligram of microsomal protein, increases in all 6 activities were evident with the treated chickens. Twofold increases were detected for APND ($P < 0.05$), ANH ($P < 0.05$), ECOD ($P < 0.01$), and AHH ($P < 0.01$) activities. The AE activity increased fourfold in treated birds ($P < 0.01$). The most pronounced increase was in ECOD, with a 15-fold increase in activity in treated birds, compared with controls ($P < 0.01$).

Significant decreases in 3 activities, expressed as per milligram of microsomal protein, were observed in the treated rats. A 40% reduction in APND ($P < 0.01$), ANH ($P < 0.01$), and ECOD ($P < 0.01$) activities developed in the treated rats. Decreases in AE, AHH, and ECOD were observed in treated rats, but these differences were not significant.

More specific changes in enzyme activities were evident for activities expressed as the turnover number for the cytochrome(s) P-450 (units/nanomole of P-450). Only AHH, AE, and ECOD activities were increased in the treated chickens at approximately 12-fold, twofold, and sevenfold, respectively. In rats, significant decreases in enzyme activities were not detected.

Discussion

Results of this study indicated that extracts of *S. drummondii* greatly affect the hepatic microsomal monooxygenase systems of chickens and rats. In White Leghorn chickens, *S. drummondii* treatment results in induction of the hepatic microsomal monooxygenase system. This finding was shown by the increases in cytochrome P-450 content, NADPH-cytochrome *c*-reductase activity, and the

TABLE 1—Effect of *Sesbania drummondii* treatment on body and liver weights in chickens and rats

	Chickens		Rats	
	Control	Treated	Control	Treated
Body weight (%) ^a	7.2 ± 2.3	6.1 ± 2.0*	4.6 ± 2.4	4.8 ± 2.8
Relative liver/body weight (% body weight) ^b	2.0 ± 0.1	2.0 ± 0.1	2.9 ± 0.3	2.8 ± 0.3

^a The results were calculated according to this formula:

$$\frac{\text{Final weight} - \text{first day weight}}{\text{first day weight}} \times 100$$

^b The results were calculated according to this formula:

$$\frac{\text{Pooled liver weight}}{\text{pooled body weight}} \times 100$$

Significantly different (Student *t* test) from control data ($P < 0.01$).
 Data are expressed as group means (± SEM; n = 3).

TABLE 3—Effect of *Sesbania drummondii* treatment on selected hepatic microsomal monooxygenase activities

	Chickens		Rats	
	Control	Treated	Control	Treated
Aminopyrine-N-demethylase (nmol of HCHO/min/mg protein) (nmol of HCHO/min/nmol of P-450)	3.09 ± 1.05 18.00 ± 3.45	6.19 ± 1.52* 17.46 ± 4.90	3.35 ± 0.18 4.78 ± 0.61	2.10 ± 0.20* 5.94 ± 1.56
Aniline hydroxylase (nmol of p-aminophenol/ min/mg protein) (nmol of p-aminophenol/ min/nmol of P-450)	2.38 ± 0.55 14.01 ± 1.32	5.86 ± 1.36* 14.06 ± 4.38	1.94 ± 0.07 2.75 ± 0.35	1.11 ± 0.06* 2.86 ± 0.64
Aldrin epoxidase (nmol of dieldrin/min/mg protein) (nmol of dieldrin/min/nmol of P-450)	0.61 ± 0.15 3.63 ± 0.99	1.85 ± 0.48* 7.49 ± 1.77*	1.56 ± 0.56 2.34 ± 0.99	0.92 ± 0.21 2.14 ± 0.33
7-Ethoxycoumarin-O-deethylase (nmol of 7-OH coumarin/ min/mg protein) (nmol of 7-OH coumarin/ min/nmol of P-450)	3.89 ± 0.45 23.19 ± 2.27	7.68 ± 0.99* 21.62 ± 3.42	3.99 ± 0.18 5.64 ± 0.76	2.39 ± 0.12* 8.70 ± 1.37
7-Ethoxycoumarin-O-deethylase (nmol of coumarin/ min/mg protein) (nmol of coumarin/ min/nmol of P-450)	8 ± 1 48 ± 14	123 ± 18* 344 ± 51*	379 ± 102 401 ± 181	88 ± 6 210 ± 31
Aryl hydrocarbon hydroxylase (nmol of 3-OH benzo(a) pyrene/min/mg protein) (nmol of 3-OH benzo(a) pyrene/min/nmol of P-450)	175 ± 45 1,024 ± 70	433 ± 17* 1,187 ± 60*	214 ± 40 305 ± 50	138 ± 6 308 ± 55

Data are expressed as group means (± SEM; n = 3). Significantly different (Student's t test) from control data (*P < 0.05, †P < 0.01).

activities of 6 cytochrome P-450-mediated enzyme activities.

The induction of the monooxygenase system in birds is generally similar to that in mammals, i.e., chickens respond to the 3-methylcholanthrene (3-MC), phenobarbital (PB), and the mixed type of classical mammalian inducers.¹⁸

3-Methylcholanthrene, β-naphthoflavone and tetrachlorodibenzo-p-dioxin (3 mammalian 3-MC type of inducers) have been examined in the White Leghorn chicken. Reported findings have included slight (twofold) increases in ethylmorphine-N-demethylase and ANH,¹⁸ moderate increases in cytochrome P-450 content,¹⁹ and large increases in ANH (7 to 20-fold) and EROD (40-fold) activities.¹⁸⁻¹⁹ Induction of NADPH-cytochrome c-reductase activity in chickens has not been reported with 3-MC inducers.¹⁸

A number of studies have examined the effects of PB on the induction of monooxygenases in chickens. Increases in cytochrome P-450 content (three- to fourfold) and increases in APND and ANH activities have been reported.^{20,21} To the best of our knowledge, the effects of PB on the chicken NADPH-cytochrome c-reductase activity have not been reported; however, in mammals, an induction of this reductase develops with PB.²²

Of the mixed-type inducers, a literature search indicated that only polychlorinated biphenyls have been studied in chickens. Arochlor 1254 and 1268 treatments result in increases in cytochrome P-450 and in the monooxygenase activities of AE, APND, and ANH.^{23,24} A change in NADPH-cytochrome c-reductase activity was not observed in chickens treated with Arochlor 1254.²³

Additionally, a number of other compounds have been determined to have inductive effects on the monooxygenase system in chickens and other birds. One such com-

pound, prochloraz, was studied by Riviere et al.¹³ in several avian species, including chickens. A fourfold increase in cytochrome P-450 with increases in EROD (threefold), AE (2.5-fold), and ANH (threefold) were observed. Govindwar et al.²⁵ examined the effects of caffeine on Sonali Pearl chicks and found slight increases in cytochrome P-450, cytochrome b₅, and NADPH-cytochrome c-reductase activities.

The response profile of the hepatic monooxygenase system of White Leghorn chickens to *S. drummondii* is somewhat different than that described above for the classical inducers. We observed essentially twofold increases in cytochrome P-450 content, APND, ANH, ANH, EROD, and NADPH-cytochrome c-reductase activities; a sixfold increase in AE; and a fifteenfold increase in EROD activity. *Sesbania drummondii* is a complex mixture of compounds, and in the chicken, may be a mixed type of inducer; however, further studies will be necessary to confirm this.

Usually compounds that are inducers of the microsomal monooxygenase systems in avian species are also inducers of the monooxygenase systems in mammals. However, this was not the case with *S. drummondii*. A depression, rather than induction, of all the activities of the hepatic monooxygenase system was observed in microsomes from the *S. drummondii*-treated rats.

Although the specific activities (nanomole of product/minute/milligram of microsomal protein) for all the substrates catalyzed by microsomes from *S. drummondii*-treated chickens were higher than those for controls, the turnover numbers (nanomole of product/minute/nanomole of P-450) were essentially the same except in the case of AE and EROD, which were three- and sevenfold higher, respectively, with microsomes from the *S. drummondii*-treated chickens, compared with microsomes from control

chickens. These data suggest induction of at least 1 new isozyme in conjunction with induction of several constitutive forms in the chicken liver. In rats, *S drummondii* appeared to suppress the constitutive isozymes and, in the case of EROD, possibly induced a novel isozyme with lower EROD activity. More rigorous proof remains to be established. At the present time it is not known whether the same component in *S drummondii* produces the effects seen in chickens and rats. The mechanisms for monooxygenase induction in chickens and depression in rats by *S drummondii* are not presently known. Whether similar responses will occur in other avian and mammalian species will require further study.

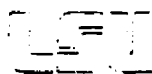
The possible implication of altering a major drug-metabolizing system by consumption of *S drummondii* could be important in any species exposed to this toxic plant. The alteration of the cytochrome P-450 system could cause less toxic agents to become more toxic, so as to produce acute intoxication on exposure. The alteration of this system will also change the pharmacokinetics of many therapeutic agents given at the time of *S drummondii* exposure. The known toxic effects of *S drummondii* in cattle presently are limited to acute toxicoses from ingestion of large quantities of seed pods. However, the potential exists for low-level chronic exposures to the plant in contaminated feed. *Sesbania drummondii* is a major weed problem in southern soybean fields. If the hepatic monooxygenase system of cattle is induced or depressed from *S drummondii*, then perhaps these changes in xenobiotic metabolism will result in ineffective or toxic drug therapies.

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January 9, 1990

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Dear Dr. Koltveit:

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21 February 1990

Marcy I. Banton, DVM
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VITA

Marcy Iva Banton was born in Cleveland, Ohio on December 31, 1956. She attended St. Mary's Dominican High School in New Orleans and graduated in May, 1974. After fulfilling pre-veterinary requirements at Louisiana State University, she gained admission to the School of Veterinary Medicine, Louisiana State University. The D.V.M. degree was conferred in May, 1981 and was then followed by one year of professional practice in Baton Rouge, Louisiana. In August 1982, she was awarded a graduate assistantship by the School of Veterinary Medicine, Louisiana State University where she pursued a Ph.D. degree in veterinary toxicology. These studies were guided by Drs. Lawrence P. Ruhr, Wayne Flory, and Charles R. Short of the Department of Veterinary Physiology, Pharmacology, and Toxicology. From January 1985 until the present, she has been employed as a research associate under the supervision of Dr. Peter L.H. Jowett in the Louisiana Veterinary Medical Diagnostic Laboratory. She was certified by the American Board of Veterinary Toxicology in July, 1989.

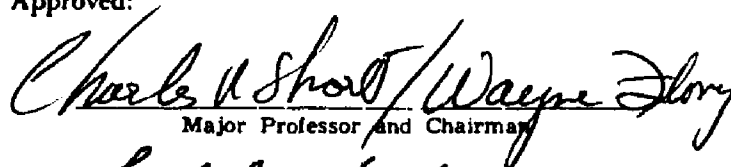
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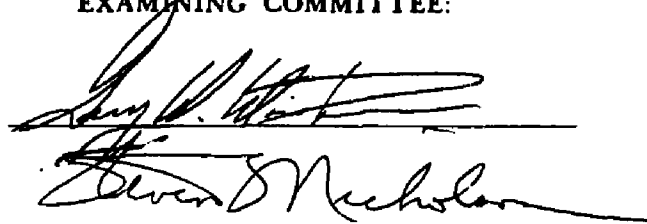
Title of Dissertation: A Comparative Study of the Effects of Sesbania drummondii on the Hepatic Cytochrome P-450 Monooxygenase Systems of Chickens and Rats

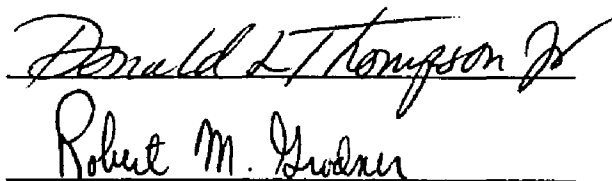
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Date of Examination:

May 7, 1990